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(54) Title: QUANTITATIVE ASSAYS EMPLOYING MAGNETIZABLE PARTICLES FOR RATE ENHANCEMENT

(57) Abstract

Assays and methods employing magnetizable particles are described where binding between a member of a specific binding pair (SBP), attached to the particle, to a solid phase surface is enhanced. The solid phase has a member of an SBP immobilized thereon, and a magnetic field is applied to attract the particles to the surface to promote a specific binding reaction. The methods may be utilized in any assay format, but are particularly useful in quantitative two-site immunometric assays.

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QUANTITATIVE ASSAYS EMPLOYING MAGNETIZABLE PARTICLES FOR RATE ENHANCEMENT

BACKGROUND OF THE INVENTION

1. Field of the Invention.

The present invention relates generally to assays for quantitatively determining the amount of an analyte in a sample. More particularly, the present invention relates to methods for enhancing the binding rate of an analyte to a specific binding partner within an immunoassay system.

A wide variety of assay techniques and methods exist for determining the amount of a target analyte in a biological or other sample. Of particular interest to the present invention, specific binding assays rely on detecting an analyte using a binding substance which reacts with the analyte in a highly selective and specific manner. The use and specificity of such specific binding assays have been greatly enhanced by the availability of monoclonal antibodies which allow the performance of immunoassays which detect the presence of particular epitope(s) on the analyte of interest in a highly specific manner.

A wide variety of specific binding assay formats exist, with assays generally being categorized based on certain performance characteristics. Assays may be competitive or non-competitive, depending on the manner in which the label is introduced to the assay system. When the label is bound within the system in direct proportion to the amount of analyte present, the assay is classified as non-competitive. The most common type of non-competitive assay is referred to as a "sandwich" assay where an immobilized binding substance, typically an antibody, captures the analyte and a second, labeled binding substance attaches to the captured analyte. In this way, the amount of label which is immobilized within the system is in direct proportion to the amount of analyte present. Usually, both the immobilized

binding substance and th labeled binding substanc are present in xcess, *i*.e. in an amount greater than the exp cted amount of analyte, so that all of the analyte will be captured and labeled, permitting a direct quantitative measure of the amount of analyte initially present in the sample.

Assays may also be categorized as quantitative or non-quantitative (also referred to as qualitative). Quantitative assays are typified by the sandwich assay described above where the amount of the label bound to a solid phase is in direct proportion to the amount of analyte initially present in a sample. Numerous non-quantitative assays exist, such as agglutination assays, where the presence of a threshold amount of an analyte results in agglutination or coalescing of particles carrying an antibody or other binding substance specific for the analyte. When agglutination is observed, presence of the analyte in the sample is confirmed. Such agglutination assays, however, are generally not very sensitive and are not suitable for determining the amount of analyte present.

Specific binding assays may also be categorized based on the label employed. Available labels include enzymes, radioisotopes, particles, and the like. Enzyme labels will usually produce a detectable signal, such as fluorescence, chemiluminescence, bioluminescence, color change, and the like. Radioactivity is normally detected by a radioactivity counter or radio-sensitive film emulsions. Particle labels may be detected based on their mass, size, and the like, or on a detectable label such as fluorescence which is incorporated within the particle.

Additionally, specific binding assays may be categorized based on the need to separate excess label from the system. Most assays are heterogeneous, where excess label must be removed from the system prior to the detection phase. Other assays are homogeneous, where the label, usually an enzyme, is activated or inhibited as a result of binding or oth r steric interaction mediated by th presence of analyte within th system. Thus, excess label need not be removed from the system prior to detection.

Of particular interest to the pr s nt invention are quantitative, heterogeneous, non-competitive assays which employ a particle or particle-associated label, where an immobilized first binding substance is present in excess to directly or indirectly capture substantially all analyte present in a sample. Such assays are often referred to as two-site immunometric assays. A second labeled binding substance, typically attached to the particle label, is reacted simultaneously with or subsequent to introduction of the sample to the solid phase. After the first binding substance has captured substantially all of the analyte which in turn has been labeled by the second binding substance, the excess label is removed, typically by washing. In order to ensure specificity and quantitative accuracy, at least the second binding substance will usually be a monoclonal antibody directed at a unique epitope on the analyte. In this way, each molecular analyte will be captured with only a single attached particle label. Thus, measurement of the amount of particle or particle-associated label will correspond directly to the amount of analyte initially present in the sample.

While generally accurate and sensitive, such quantitative, heterogeneous, non-competitive assays often require a substantial amount of time to run, typically tens of minutes or longer. This time is necessary to permit binding of the analyte to the immobilized first binding substance. It will be appreciated that in virtually all solid phase assays, the binding kinetics are slow because of diffusional limitations on binding to the solid phase. Use of a particle label can further hinder the binding kinetics since the particles (which are large compared to the molecular species involved) will move comparatively slowly through the liquid phase of the assay system. Such a delay in assay completion time is undesirable, particularly in instances where the assay results are necessary for making immediate medical decisions. For example, the treatment of patients suspected of suffering from heart attacks will frequ ntly depend on the detection of certain cardiac markers, such as troponins, creatine kinase isozymes, such as CKMB, and the like. For such patients, the

proper treatm nt should begin as soon as possibl and any delay in diagnosis is undesirable.

For these reasons, it would be desirable to provide rapid assay formats, particularly in the case of non-competitive heterogeneous assays employing particle labels. It would further be desirable to provide methods for enhancing the binding rate of analytes to a specific binding partner immobilized on a solid phase, particularly when the analyte is labeled with a particle label. It would further be desirable if such assays and methods would also find use in competitive, heterogeneous, non-quantitative and/or other assay systems, and which may further employ other labels in addition to particle labels.

2. Description of the Background Art.

U.S. Patent 5,318,914, describes assays involving magnetically accelerated binding of particles to a solid phase surface. EP 351 857 describes a non-quantitative assay for detecting substances in a sample based on the geometric distribution of particle-bound substances across the bottom of a conical or spherical test well. In one embodiment, precipitation of the particles is magnetically enhanced. Magnetizable particles and assay formats are described in U.S. Patent Nos. 4,297,337; 4,554,088, 4,661,408; 4,672,040; 4,731,337; and 4,745,077; PCT publication WO 89/0473; British Patent 2,152,664; and Japanese Patent Publications 3189560 and 63108264.

SUMMARY OF THE INVENTION

Assays for determining the presence of an analyte which is a member of a specific binding pair (SBP) in a sample comprise exposing the sample to a solid phase surface having a reaction zone including one member of an SBP in the presence of magnetizable particles having one member of the same or a different SBP bound thereto. The specificity of the various SBP members will be such that the analyte will bind directly or indirectly to the solid phase. A magnetic field is applied to attract the magnetizable particles to the reaction zone,

where the magnetizable particles and/or particle-associated label become bound in a manner which depends on the amount of analyte initially in the sample. Usually, the amount of analyte is determined based on the amount of magnetizable particles bound to the reaction zone, typically by interferometrically or spectrophotometrically determining the thickness of a uniform layer of particles deposited across the reaction zone. Alternatively, binding of a separate label (not associated with the magnetizable particles) to the particles can be mediated based on the amount of analyte, and the magnetizable particles may be captured within a reaction zone which specifically binds all particles or a certain group of particles without regard to the amount of analyte originally present.

The assays may find particular use with non-competitive, heterogeneous assay formats where the magnetizable particle also acts as a detectable label. In such formats, the analyte will be a ligand and the SBP member in the reaction zone will be anti-ligand. The magnetizable particles will also have anti-ligand bound thereto, so that the magnetizable particles become bound within the reaction zone in a non-competitive, sandwich format. Usually, the anti-ligand bound to the magnetizable particles will be specific for a unique epitope on the analyte ligand so that only one particle will be bound to the solid phase surface for each unit, usually a molecule, of analyte. The magnetizable particles may then be detected directly, *i.e.* based on their mass or size. Alternatively, the magnetizable particles can be coupled to other labelling substances, such as enzymes, radioisotopes, fluorescent materials, or the like, to permit their detection.

The assays may also find particular use in competitive and non-competitive, heterogeneous assay formats where one or more initial assay steps result in a population of magnetizable particles, wherein individual particles may vary in a manner which results from the presence of analyt in the sample. The entire population of magnetizable particl s (or some fraction thereof which is not dependent on the

presence of analyte) is then specifically bound within the reaction zone.

The assays of the present invention may also find use in competitive assay formats and/or homogeneous assay formats. In a first competitive assay format, the analyte is a ligand and the reaction zone comprises anti-ligand. The magnetizable particles have a ligand or ligand analogue bound thereto, and the magnetizable particles are thus competitively bound within the reaction zone in inverse proportion to the amount of native ligand analyte in the sample. In an alternative competitive assay format, the analyte is ligand and the reaction zone comprises ligand or ligand analogue. The magnetizable particles have anti-ligand bound thereto, whereby the magnetizable particles are competitively bound within the reaction zone in inverse proportion to the amount of ligand initially present in the sample.

In preferred aspects of the quantitative assays of the present invention, the magnetic field is interrupted to permit removal or separation of non-bound magnetizable particles from the reaction zone prior to determining the amount of analyte in the sample. In an optional aspect, a second magnetic field is applied to the sample from a location remote to the reaction zone in order to draw non-bound particles away from the reaction zone. The solid phase surface on which the member of the SBP is bound is preferably flat so that the magnetizable particles will bind in a substantially uniform thickness thereacross.

The present invention further provides an assay for determining the presence of an analyte which is a member of a SBP where the sample is exposed to a solid phase surface having a reaction zone comprising one member of a SBP in the presence of magnetizable particles having one member of at least two different SBP's bound thereto. A first particle-bound SBP member binds directly or indirectly to the solid phase SBP member and a second particle-bound SPB member binds directly or indirectly to the analyte. By incubating the magnetizable particles with the sample for a time sufficient to permit binding between the second particle-bound SBP member

and the analyte to occur, efficient capture of the analyte onto the particles can be achieved. By then applying a magnetic field across the reaction zone, the rate of migration of magnetizable particles to the reaction zone can be enhanced. Thus, a constant amount of the magnetizable particles (which are usually present in excess in order to capture substantially all analyte) will be rapidly bound within the reaction zone to immobilize the analyte. The presence of analyte within the reaction zone may then be detected by variety of conventional means, typically relying on detection of label bound to the analyte. Usually, label will be bound to the analyte through use of a labelled SBP member which binds to the analyte. The labelled SBP member is exposed to sample prior to applying the magnetic field. The label is then captured together with the analyte within the reaction zone. In the exemplary embodiment, the reaction zone SBP is biotin and the first particle-bound SPB member is streptavidin. The second particle-bound SPB member will usually be anti-analyte capable of directly binding the analyte.

The present invention further provides an improved non-competitive binding assay of the type where an analyte is bound to a solid phase surface by a first antibody and detected by a labelled second antibody specific for a monomeric epitope on the analyte. The improvement comprises employing a magnetizable particle as a label, and applying a magnetic field aligned with the reaction zone on the solid phase to enhance the binding rate of analyte to the first antibody.

In a particular aspect of the method of the present invention, an analyte is determined in a sample by first exposing the sample to magnetizable particles having a SBP member bound thereto. The magnetizable particles are able to bind to the analyte in a liquid phase directly or indirectly via the SBP member. The magnetizable particles are then separated from the non-bound analyte by applying a magnetic field which permits removal of the non-bound analyte. The magnetizable particles are then resuspended in a reaction

medium. The reaction medium is exposed to th solid phase, and SBP in a reaction zone on the solid phase binds directly or indirectly to the analyte which has been previously bound to the magnetizable particles. A magnetic field aligned with the reaction zone is applied to attract the magnetizable particles thereto. The presence of analyte in the samples is then determined based on the binding of magnetizable particles to the reaction zone in a manner which is mediated by the presence of analyte in the sample.

In another specific aspect of the present invention, an assay for determining the presence of at least two analytes in a sample comprises reacting the sample with first magnetizable particles bound to (1) a first SBP member which binds directly or indirectly to a first analyte and (2) a first capture receptor. The sample is sequentially or simultaneously reacted with second magnetizable particles bound to (1) a second SBP member which binds directly or indirectly to a second analyte and (2) a second capture receptor. The sample is then exposed to a solid phase having a first reaction zone comprising first capture ligand which binds to the first capture receptor and a second reaction zone comprising second capture ligand which binds to the second capture receptor. A magnetic field aligned with the reaction zones is then applied to attract the magnetizable particles thereto. The presence of the first and second analytes is determined based on the binding of said analytes within their respective reaction zones. Preferably, the magnetic field is periodically disrupted to permit non-specifically bound magnetizable particles to migrate in a direction which is not aligned with the magnetic field. Such migration facilitates movement of the magnetizable particles so that they can bind within their intended reaction zones.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A-1D are schematic illustrations of a first assay system employing immobilized antibody for capturing analyte in the reaction zone. Labelled antibody is employed for labelling the captured analyte in the liquid phase, and

binding of the analyte-antibody-particl complex to the immobilized antibody is magnetically enhanced, as illustrated in Fig. 1C. Separation of non-bound magnetizable particles is magnetically enhanced, as illustrated in Fig. 1D.

Figs. 2A-2D are schematic illustrations of a second assay system employing different immobilized capture antibodies in two reaction zones. The assay format is generally the same as that described for Figs. 1A-1D, except that different analytes are bound and labelled within each reaction zone.

Figs. 3A and 3B illustrate a first optional competitive assay format where analyte or an analyte analogue is bound within a reaction zone and where native analyte and immobilized analyte or analyte analogue compete for binding to magnetizable particle-labelled antibody complex to the analyte.

Figs. 4A and 4B illustrate a second optional competitive assay format where antibody to the analyte is immobilized in a reaction zone. The immobilized antibody competes for binding to native analyte and magnetizable particle-labelled analyte or analyte analogue.

Figs. 5A-5C illustrate a third optional assay protocol according to the present invention where bound magnetizable particles are separated from non-bound analyte in a magnetic field prior to specific binding of analyte-bound magnetizable particles within a reaction zone.

Figs. 5D-5F illustrate a fourth optional assay protocol according to the present invention where multiple analytes are captured via receptor-ligand pairs to specific reaction zones on a solid phase. The amount of analyte found within each reaction zone is determined based on the presence of a conventional label.

Figs. 5G-5I illustrate a method for immobilizing different capture antigens within different reaction zones via capture receptor-ligand binding pairs on a solid phase.

Fig. 6A illustrates an exemplary solid phase which may be employed in the assay methods of the present invention.

The solid phase is a disk having a plurality of reaction zones formed on a flat face thereof.

Fig. 6B illustrates the disk solid phase of Fig. 5 adjacent to a magnet for applying a magnetic field in accordance with the principles of the present invention.

Figs. 7 and 8 illustrates an experimental apparatus used for performing the magnetically enhanced assay protocols described in detail in the Experimental section herein.

Figs. 9-11 are graphs illustrating the results of examples described in detail in the Experimental section hereinafter.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Assays and methods are provided for detecting the presence of (usually quantitatively measuring the amount of) an analyte present in a sample and in particular for enhancing the binding rate of the analyte to a solid phase surface in an assay system. The analyte will be a member of a specific binding pair (SBP), including compounds, compositions, aggregations, and virtually any other substance which may be detected or reacted by immunological or equivalent techniques. That is, the analyte, or a portion thereof, will be antigenic or haptenic, defining at least one epitopic site, or will be a member of a naturally-occurring binding pair (*e.g.*, carbohydrate and lectin, hormone and hormone receptor, ligand and anti-ligand, and the like). Analytes of particular interest include antigens, antibodies, proteins, glycoproteins, carbohydrates, macromolecules, toxins, bacteria, tumor markers, and the like, which define a plurality of epitopic sites. Other analytes of interest include haptens, drugs, and other small molecules, which usually define only a single epitopic binding site. A non-exhaustive list of exemplary analytes is set forth in U.S. Patent No. 4,366,241, at column 19, line 7, through column 6, line 42, the disclosure of which is incorporated herein by reference. Of particular interest to the present invention is the detection of cardiac markers, such as troponin I, troponin T, myoglobin, and creatine kinase isoenzymes.

The analytes may be present in a wide variety of samples, where the sample is liquid, can be liquified, or can be suspended in a liquid. The methods of the present invention will find their greatest use with biological specimens such as blood, serum, plasma, urine, cerebral fluid, spinal fluid, ocular lens liquid (tears), saliva, sputum, semen, cervical mucus, scrapings, swab samples, and the like, which are frequently employed in the diagnosis and monitoring of disease and therapeutic treatments. In addition, the methods of the present invention may be used with industrial, environmental, and food samples, such as water, process streams, milk, meat, poultry, fish, conditioned media, and the like. In certain circumstances, it may be desirable to pretreat the sample, such as by liquification, separation, solubilization, concentration, filtration, chemical treatment, or a combination of these steps, in order to improve the compatibility of the sample with the remaining steps of the assay, as described hereinafter. The selection and pretreatment of biological, industrial, and environmental samples prior to immunological testing is well known in the art and need not be further described.

The assays and methods of the present invention rely on use of a magnetic field to immobilize a magnetizable particle within a reaction zone on a solid phase surface where a member of a specific binding pair (SBP) on the particle binds directly or indirectly to an SBP member in the reaction zone. Usually, the amount of bound particles is mediated by the presence of analyte in the sample. Alternatively, all magnetizable particles may be immobilized and bound via the SBP members, and the presence and/or amount of analyte determined by a separate label which is introduced into the system in an analyte dependent manner. Such binding will be effected through at least one specific binding reaction, usually through at least two binding linkages, and frequently through three or more specific binding linkages, depending on the particular assay format. In the case of non-competitive, sandwich assay formats, a binding substance which directly or indirectly captures the analyte will be immobilized in the

reaction zone on the solid phase surface. The magnetizable particle will be selectively bound to the captur d analyte through one or more members of specific binding pair(s). Competitive assay formats will often rely on direct, competitive binding of magnetizable particles through a linkage comprising only one member of a specific binding pair, such as the analyte itself, an analyte analogue, or an antibody to analyte.

A "specific binding pair" will include two members, at least one of which is a macromolecular compound having spatial and polar features which permit specific binding to the other compound, which may be another macromolecule or a small molecule. SBP members useful in the present invention will be selected or prepared to specifically bind to analyte, typically being antibodies raised against the target analyte. Other specific binding pairs which may be employed for direct or indirect binding within the assay systems of the present invention include lectins and carbohydrates, hormones and hormone receptors, enzymes and enzyme substrates, biotin and avidin, vitamins and vitamin-binding proteins, complementary polynucleotide sequences, drugs and receptors, enzymes and reaction products, and the like. Biotin and avidin derivatives may also be used, including biotin analogues, streptavidin, and the like.

Most often, no known natural specific binding substance will exist, and one will have to be prepared. For antigenic and haptenic analytes, antibodies may be prepared by well-known techniques. In particular, antibodies and monoclonal antibodies may be prepared by well-known techniques involving the immunization of vertebrates with an immunogen comprising an epitopic site of interest. Such techniques are well described in the literature. See, for example, *Antibodies: A Laboratory Manual*, Harlow and Lane, eds., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988.

The assays and methods of the present invention will usually rely on detection of a label which becomes immobilized within the reaction zon in an amount which d pends on the amount of analyte initially present in the sample. The label

may be the magnetizable particle which is bound to a member of an SBP which binds directly or indirectly to the reaction zone. The magnetizable particle can be detected by any conventional means capable of detecting particles based on size, mass, and optical characteristics, such as light absorbance, light scattering, change in refractive index, turbidity, fluorescence, chemiluminescence, and the like. The methods of the present invention will be particularly suitable for assays where the thickness of the magnetizable particle layer is detected using interferometry, light scattering, and/or light absorbance. For example, assay protocols employing an interferometer are described in copending application Serial No. 08/086,345 and PCT US94/07184, the disclosures of which are incorporated fully herein by reference. Assays based on detecting changes in optical characteristics are described in Collet-Cassert et al. (1989) Clin. Chem. **35**:141-143 and Hadfield et al. (1987) J. Immunol. Meths. **97**:153-158.

Alternatively, other detectable labels may be employed in the assay systems of the present invention. Conventional labels include a variety of compounds, molecules, moieties, and the like, which can be bound to the magnetizable particle or other substance whose binding is mediated by the presence of analyte and which can be detected in a suitable assay protocol. Exemplary labels include enzymes, enzyme cofactors, enzyme inhibitors, radioisotopes, chemiluminscent compounds, chromophores, scintillants, and the like. Such labelling systems may further include intermediate binding substances which are themselves members of an SBP as described above. For example, avidin and biotin may be attached to the magnetizable particle or other binding substance to mediate binding of a label, particularly in the case of non-competitive assay protocols. Many labels will be visually or optically detectable, such as chromophores, lumiphores, fluorophores, scintillants, chemiluminescers, as well as those which mediate the formation of a product that can be observed visually, *e.g.*, a dye. Lumiphores, fluorophores, and chemiluminescers may be visualized directly through

spectroscopic means while enzyme reporter molecules usually require addition of a substrate to form a reaction product that will be visible on the solid phase surface. Such labelling materials and systems are well known and amply described in the patent and scientific literature.

Exemplary assay protocols according to the present invention will be two-site immunometric assays, often referred to as sandwich assays, which utilize a pair of antibodies specific for the target analyte. One of the antibodies will be immobilized on the solid phase surface, and both of the antibodies will be present in excess over the amount of target analyte suspected of being present in the sample. Preferably, the liquid phase antibody will be directly attached to a magnetizable particle and will be specific for a unique epitopic site on the analyte. In this way, only a single magnetizable particle will become attached to the analyte. The nature of the solid phase (capture) antibody is less critical. Generally, the combination of analyte, liquid phase antibody, and magnetizable particles is sufficiently large so that only a single aggregate can become bound at any antibody site on the solid phase. Moreover, since solid phase antibody will be present in excess, two or more antibodies may bind to the analyte without adversely affecting the quantitative accuracy of the assay.

Such two-site immunometric assays can be performed with either simultaneous or sequential addition of sample and magnetizable particle-labelled antibody. So long as binding of the particle and analyte does not interfere with subsequent binding to the capture antibody or other binding substance, it does not matter if the magnetizable particle binds analyte in the liquid phase or at or near the solid phase surface. In either case, application of the magnetic field will enhance migration of the magnetizable particle toward the solid phase surface, where binding of the analyte to the capture antibody and/or of the magnetizable particle-antibody complex to the analyte will occur.

Such two-site immunometric assays will require a separation or wash step after the initial binding of

magnetizable particles to the solid phase surface. It will appreciated that excess magnetizable particles will be pres nt which will need to be removed prior to detection. Such separation may be achieved by conventional washing using a buffer or other suitable liquid. In a specific aspect of the present invention, such washing step may be magnetically enhanced using a magnet which is disposed away from the reaction zone to attract the excess magnetizable particles.

After the non-specifically bound magnetizable particles have been removed from the reaction zone, the amount of analyte in the sample may be quantitatively determined based on the amount of magnetizable particles bound within the reaction zone. For example, those assays of the present invention which result in a binding of magnetizable particles in an amount mediated by the presence of analyte in the sample may be read by measuring that amount by any of a variety of methods, including interferometry, spectrophotometry, scatterometry, or the like, as described generally above.

In a second preferred aspect of the present invention, the magnetizable particles will be utilized to capture all or a constant (repeatable) amount of the analyte present in a sample onto a solid phase reaction zone. Such assays may be run generally as described above, except that the reaction zone will comprise a member of a SBP which binds to a first SBP member bound to all magnetizable particles. Thus, binding of the magnetizable particles to the reaction zone will not be mediated by the presence of analyte in the sample. Preferably, the reaction zone SBP member and first particle-bound SBP member will have a high binding affinity, more preferably being avidin (or streptavidin) and biotin, respectively, which have a binding affinity in excess of $10^{-14}M^{-1}$. Moreover, by providing multiple biotin or avidin moieties on the magnetizable particle or the solid phase, binding avidity between the particles and the solid phase will be even higher. High binding avidity is desirable to keep the particles bound during subsequent wash and other steps of the assay. It would also be possible to use other binding pairs having very high affiniti s, such as antibodies to highly

immunogenic haptens, such as fluorescein, dinitrophenol (DNP), luminol, and the like.

A second SBP member is also bound to the magnetizable particle where the second particle-bound SPB member binds directly or indirectly to the analyte. In most cases, the second particle-bound SPB member will be an antibody directed against the analyte of interest. It would also be possible to use known intermediate binding substances, such as avidin, biotin, antibodies against antibodies derived from a particular animal source (e.g., anti-mouse antibodies), and the like.

Such assays will preferably employ excess second particle-bound SBP so that all analyte present in the sample may be captured onto the solid phase. In such cases, however, the particle itself cannot serve as the label since the amount of bound particle will not be mediated by the presence of analyte in the sample. Instead, such assays will usually rely on introduction of a label to the analyte, typically by means of a labelled SBP member reacted with the analyte prior to application of the magnetic field. In this way, a reaction between the magnetizable particles and both the analyte and the SBP-label may occur in the liquid phase, preferably while the particles and/or sample are being agitated to enhance the reaction rate. Once the reaction has been completed, the rate of binding of the particles to the reaction zone may be magnetically enhanced, as generally described above.

Such protocols are particularly useful for simultaneously detecting multiple analytes in a single sample. The solid phase will include multiple reaction zones capable of binding different groups of magnetizable particles. For example, each group of magnetizable particles may comprise a specific capture ligand, such as biotin, fluorescein, DNP, and luminol, and the reaction zones may comprise the corresponding capture receptor, such as avidin (or anti-biotin antibody), anti-fluorescein, anti-DNP, and anti-luminol, respectively. Each group of magnetizable particles will further comprise an SBP member specific for a targ t analyte of interest. The magnetizable particles thus become bi-functional or bi-

specific reagents which are able to enhance the binding rate of each target analyte to a specific reaction zone on the solid phase. In order to permit lateral migration of the particles so that all or a predictable proportion of the particles will bind within the corresponding reaction zone, the magnetic field will be periodically interrupted to permit particles which have been magnetically attached to unintended regions on the solid phase to move toward their intended reaction zones.

The magnetic field can be interrupted in a variety of ways. When using a permanent magnet, the field may be interrupted by moving the magnet away from the solid phase. Electromagnets can be interrupted by movement or termination of power. In either case, the liquid phase of the assay will preferably be agitated while the magnetic field is disrupted to enhance migration of the non-specifically-bound particles over the solid phase surface. Several cycles of applying the magnetic field followed by agitation of the liquid phase will enable a high degree of specific bind of particles within their intended reactions.

The assays and methods of the present invention can use a variety of magnetizable particles, typically having size in the range from 0.025 μm to 2 μm, preferably 0.1 μm to 1 μm. The particles may be composed of organic polymers, ceramics, glasses, gels, or the like. Preparation of magnetizable particles is well described in the scientific and patent literature. See, for example, U.S. Patent Nos. 4,297,337; 4,554,088; 4,661,408; 4,672,040; and 4,731,337, the disclosures of which are incorporated herein by reference. Such magnetizable particles will include a magnetic substance, typically an iron oxide or chromium substance, which will be attracted in a magnetic field.

Methods for attaching such magnetizable particles to members of a specific binding pair, such as an antibody, are also well known and described in the patent and scientific literature. Such attachment will usually be achieved through covalent bonding using conventional chemistries depending on the nature of the particle. Frequently, binding will be

achieved using a bivalent cross-linking substance which is capable of attaching at one end to a member of the specific binding pair and at the other end to the magnetizable particle. Such substances include carbodiimides, glutaraldehydes, and diazotization coupling.

The solid phase surface employed in the methods of the present invention will include at least a single reaction zone, often including two or more reaction zones, frequently including from 2 to 100 reaction zones, and often including from 4 to 50 reaction zones. The reaction zones may be specific for a single analyte, or different reaction zones may be specific for different analytes. It will be appreciated, of course, that the reaction zones may be capable of directly or indirectly, competitively or non-competitively binding, or providing any other type of binding compatible with the assay formats of the present invention.

The reaction zones are preferably flat to enhance uniformity of magnetizable particle binding thereacross. The reaction zones will usually have a minimum area of 0.1 mm^2, usually being from 0.5 mm^2 to 100 mm^2, and preferably from 1 mm^2 to 50 mm^2. In an exemplary embodiment, a plurality of circular reaction zones having areas in the range from 0.5 mm^2 to 100 mm^2 will be formed on a single flat surface of a disk substrate.

The substrates which provide the solid phase surface may be composed of a variety of conventional materials, including organic polymers, glass, ceramics, metals, and the like. For use with interferometers, substrates will preferably be transparent or translucent, and will be composed of organic polymers. An exemplary material is methylmethacrylate polymer.

The magnetic field may be applied by a permanent or electromagnetic source. The strength of the magnetic field should be suitable to enhance the migration rate of the magnetizable particles through the sample or reaction media by a factor of at least two, preferably at least five, and usually by 10, or more. Suitable magnetic fluxes will be in

the range from 2500 gauss to 15,000 gauss, measured within the reaction zone.

An advantage of the present invention is that the volume of sample is not critical. Depending on the number and size of reaction zones, the total sample volume may be as low as 0.001 ml or as large as several ml, usually being in the range from 0.01 ml to 0.5 ml, more usually being in the range from 0.02 ml to 0.1 ml.

Referring now to Figs. 1A-1D, an assay system 10 comprises a solid phase 12 having a reaction zone 14 on a surface thereof. A wall 16 is formed about the periphery of the solid phase 12 to define a receptacle volume 18 capable of receiving a liquid sample S. It will be appreciated that the wall 16 may be formed as part of the solid phase 12, or may be formed separately so that the wall can be removed to facilitate detection of magnetizable particles on the solid phase 12, as described in more detail hereinafter. A first magnet 20 is disposed adjacent to the reaction zone 14 and extends across substantially the entire area of the reaction zone. A second magnet 22 is disposed adjacent the wall 16. The magnets 20 and 22 may be permanent magnets but will more usually be electromagnets which are capable of being energized to apply a magnetic field and de-energized to remove the magnetic field.

The sample S is added to the receptacle volume 18 and will be expected to include analyte A therein. The reaction zone 14 includes a plurality of anti-analyte molecules 24, usually antibodies, which are capable of directly binding the analyte. As explained above, however, in other protocols, the member of a SBP immobilized within the reaction zone 14 may be capable of indirectly binding the analyte only, *e.g.*, through an avidin-biotin-antibody linkage. A second anti-analyte 26 bound to a magnetizable particle 28 is added to the sample, either prior to the time the sample is introduced to the receptacle volume 18 or subsequent to such sample addition. In either case, the antibody 26 will bind specifically to the analyte A, usually while both are present in th liquid sample. Some binding of analyte and/or analyte

bound to antibody 26 will occur to the immobilized antibody 24, as illustrated in Fig. 1B. The size of the magnetizable particle 28 coupled to the antibody 26 and analyte A, however, will inhibit migration toward the immobilized antibody 24. Thus, the time required to achieve complete binding of the analyte may be excessive for many applications. In order to enhance the binding rate, a magnetic field is applied from magnet 20 to attract magnetizable particles 28 toward the reaction zone 14. Once the magnetizable particles 28 are in proximity to the reaction zone 14, the immobilized antibodies 24 will be able to bind to the analyte which is bound to the antibodies 26. The result is immobilized analyte, antibody, and magnetizable particle, as illustrated in Fig. 1C. Excess antibody 26 bound to magnetizable particles 28 may then removed by washing, optionally with the application of a magnetic field from magnet 22 which draws the excess antibody and magnetizable particles to the side, as illustrated in Fig. 1D. The sample may then be withdrawn, the solid phase surface washed, and the magnetizable particle binding then determined using any suitable means, such as the interferometric measurement described in copending applications Serial No. 08/086,345 and PCT US94/07184, the disclosures of which have previously been incorporated herein by reference.

Referring now to Figs. 2A-2D, an alternative assay system 40 comprising a first reaction zone 42 and a second reaction zone 44 on a solid phase 46 is illustrated. The first reaction zone 42 includes a first antibody 50 immobilized therein which binds to a first analyte A1. A second antibody 52 is immobilized in the second reaction zone 44 and binds to a second analyte A2. A first magnet 56 is provided adjacent the two reaction zones 42 and 44 and a second magnet 58 is provided around the periphery of the reaction zones.

The system 40 may be used to simultaneously detect the presence of both analytes A1 and A2, as illustrated in Figs. 2B-2D. After addition of magnetizable particle-labelled antibody 60 specific for analyte A1 and magnetizable particle-

labelled antibody 62 specific for antibody for A2, the situation will be as illustrated in Fig. 2B. Binding will occur among the antibodies, analytes, and capture antibodies 50 and 52, respectively. The capture of analytes and magnetically-labelled antibodies is enhanced by applying a magnetic field from the magnet 56 adjacent the reactions zones, as illustrated in Fig. 2C. Separation of the magnetizable particle-labelled antibodies 60 and 62 may be enhanced using peripheral magnet 58, as illustrated in Fig. 2D. Referring now to Figs. 3A and 3B, a possible competitive assay format employing the magnetic enhancement of the present invention is illustrated. An analyte or analyte analogue 100 is immobilized on a solid phase surface 102. Analyte A in a sample is combined with magnetizable particle-labelled antibody specific for the analyte. Competitive binding occurs between the native analyte A in the liquid phase and the immobilized analyte or analyte analogue 100 on a solid phase 102. Binding of the magnetizable particle-labelled antibody 104 to the immobilized analyte or analyte analogue 100 is enhanced by a magnetic field from magnet 110. It will be appreciated that the strength of the magnet field and timing of when the magnetic field is applied must be carefully selected so that the distribution of antibody 104 between liquid phase native analyte and immobilized analyte is not disrupted.

A second competitive assay format is illustrated in Figs. 4A and 4B. There, antibody 200 specific for analyte A is immobilized on a solid phase 202. Analyte or analyte analogue 204 bound to a magnetizable particle 206 is introduced to the liquid phase, where the analyte 204 and native analyte A compete for binding to the immobilized antibody 200. Again, application of a magnetic field from magnet 210 will enhance the rate of binding of the magnetizable particle 206-analyte 204 conjugate which might otherwise be unduly hindered by the size of the magnetizable particle.

Referring now to Figs. 5A-5C, an assay protocol for detecting analyte A with antibody 500 bound to magnetizable

particles 502 is illustrated. The antibodies 500 are introduced to the sample medium and allowed to react with the analyte A. The reaction may be direct, i.e. the antibody having covalently bound magnetizable particles 502 may react directly with the analyte A. Alternatively, various indirect binding protocols may be provided as discussed above. After the antibody 500 and analyte A have reacted for a time sufficient to permit binding between the antibody and analyte, as illustrated in Fig. 5A, a magnet 510 will be applied to a portion of the reaction vessel 512 remote from a reaction zone having capture antibody 504. This magnetic separation step will separate unbound analyte A from that portion of the analyte which is bound to antibodies 500, as illustrated in Fig. 5B. After removing the sample solution and washing the magnetically immobilized antibodies 500, the antibodies bound to analyte A are resuspended in a reaction medium 514, as illustrated in Fig. 5C. A magnetic field from a second magnet 516 is then applied in a manner which is aligned with the reaction zone comprising capture antibodies 504. The second magnetic field induces migration of the magnetizable particles 502 bound to antibody 500 which in turn are bound to the analyte A so that the analyte is captured onto the antibodies 504. Presence of the analyte bound to the capture antibody may then be detected, based either on the magnetic particle label or on a separate label which can be introduced by any conventional means, as discussed hereinbefore. Such protocols, where most or all analyte is immobilized in a first capture step, are advantageous since they avoid the well known "hook" effect and can avoid problems associated with the presence of interfering substances initially present in the sample.

Referring now to Figs. 5D-5F, a preferred assay protocol according to present invention utilizes a solid phase 600 having a plurality of reaction zones, each of which comprises a high binding affinity ligand or hapten. For example, first reaction zone might include biotin B, a s cond reaction zone might include fluorescein F, and a third reaction zone might include dinitrophenol D, as illustrated.

The reaction zones are us d to detect each of three analytes, illustrated as antigens Ag_1, Ag_2, and Ag_3. Bifunctional magnetizable particles are introduced to the reaction medium which contains the analyte. The first bifunctional particle includes the magnetizable particle, an antibody capable of binding antigen Ag_1, and a receptor capable of binding the biotin, typically avidin. Such a bifunctional particle is shown as $\alpha B \bullet Ab_1$. The biotin binding component could also be steptavidin or anti-biotin antibody. Similar bifunctional magnetizable particles $\alpha F \bullet Ab_2$ and $\alpha D \bullet AB_3$ are provided for binding to the analytes Ag_2 and Ag_3, respectively. The fluorescein binding component will typically be anti-fluorescein antibody (αF) and the dinitrophenol binding component will typically be anti-dinitrophenol antibody (αD). The bifunctional magnetizable particles are introduced to the reaction medium, as shown in Fig. 5D, and react to form magnetizable particle-containing complexes, as shown in Fig. 5E. Binding of the magnetizable particle-containing complexes can then be promoted by applying magnetic field using magnet 620, as illustrated in Fig. 5F.

The assay protocol illustrated in Fig. 5D-5F is useful for capturing substantially all or a constant amount or portion of antigen present in a sample within a corresponding reaction zone. To do so, the bifunctional magnetizable particles will usually be introduced in excess and sufficient binding ligand will provided in the corresponding reaction zones to capture a constant amount of the particles therein. Thus, the amount of analyte bound within the reaction zone cannot be determined based on the amount of magnetizable particles introduced. Instead, conventional labelling systems may to be introduced which bind to each of the analytes. As illustrated in Fig. 5E, labelled antibodies Ab_1*, Ab_2*, and Ab_3*, may be introduced to specifically introduce label on each of the corresponding analytes. The labels may be any of the conventional labels discussed above and may be detected spectrophotometrically, radiometrically, luminescently, fluorescently, or the like.

Referring now to Figs. 5G-5I, yet another assay protocol according to the pres nt inv ntion is illustrated. A solid phase surface 700 includes receptors B, F, and D, generally as described for solid phase 600. In order to derivatize each of the reaction zones so that they can specifically bind to a target analyte, antibodies are introduced to the reaction zones using the ligand binding partner for each of the receptors. For example, antibody Ab_1 specific for analyte Ag_1 may be introduced to the first reaction zone by coupling avidin or other anti-biotin substance to the antibody and reacting, as illustrated in Fig. 5G. Similarly, antibody Ab_2 to analyte Ag_2 can be introduced using an anti-fluorescein conjugate, and antibody Ab_3 to analyte Ag_3 can be introduced to the third reaction zone using anti-dinitrophenol (αD). Presence of each of the antigens may then be determined using either the magnetizable particle as a label or a separate label which can be introduced to the system, as generally described above.

Referring now to Figs. 6A and 6B, an exemplary solid phase 300 is a disk having a plurality of reaction zones 302, 304, and 308, disposed on a flat surface thereof. Each of the plurality of reaction zones 302, 304, and 308, are disposed in rings, where the individual zones are circumferentially spaced-apart. Reaction between analyte in the reaction zones is enhanced by an annular magnet which is disposed adjacent to the bottom size of the disk 300, as illustrated in Fig. 6B. It will be appreciated, of course, that a separable wall will be provided around the periphery of the solid phase 300 in order to contain a liquid sample, as generally described above. The wall may be removed and the solid phase of substrate 300 washed after the reaction has been completed. The disk substrate 300 is particularly suitable for use in the interferometer measurement system described in copending application Serial No. 08/086,345, the disclosure of which has previously been incorporated herein by reference. It will be appreciated, however, that the disk and other substrates according to the present invention can be used in any measur ment syst m that can detect the presence and/or amount

of particle (or other label associated with the particle) which has been bound to the substrate in a magnetically enhanced manner.

The following examples are offered by way of illustration, not by way of limitation.

EXPERIMENTAL

All experiments were performed in duplicate and included negative controls (i.e., no CKMB present). Experiments were performed with varying levels of CKMB to obtain a dose response.

A. Materials and Methods

1. DISK SUBSTRATES

Acrylic discs were obtained from Germanow-Simon (Hesalite HT: crosslinked, uv-stabilized, and heat resistant). The discs had a diameter of 35 mm, a thickness of 0.6 mm, and a 2.4 mm diameter hole in the center. Coating solutions (as described below) were dispensed to create 32 spots spaced equidistant at a 1-cm radius. The volumes were applied with an "Easy Step" dispenser from Tri-Continent Scientific. This was performed by contacting the syringe tip of the dispenser to the acrylic surface. Fluid volumes were dispensed by positive displacement with the syringe plunger. This resulted in spots, each having a diameter of approximately 1 mm, with a spot center-to-center spacing of approximately 2 mm. After the spots were allowed to dry, the discs were washed several times with deionized water.

2. PREPARATION OF ANTI-CKMB/ANTI-MURINE IgG COATED MAGNETIZABLE PARTICLES

100 µL of carboxylate modified microspheres (Bangs Labs, #M0007700CN, 0.77 µm diameter, latex encapsulated, 41% magnitite) in a 10% wt./vol. suspension was added to 1.0 mL of PBS, pH 5.5 buffer. The magnetizable particles were pelleted to the bottom of a test tube with a magnet (Polysciences, cat #19772), and the supernatant was removed. A solution (2 ml) containing 1 mg/mL of anti-murine IgG (Sigma #M3534) in PBS pH 5.5 was added to the particles and

then sonicated in a water bath sonicator for 10 minutes to assure dispersal of the particles. 40 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Pierce, EDC #22980) was added to the particle suspension. The mixture was allowed to react for 2 hours at room temperature with constant mixing and a 5 minute sonication step every 30 minutes to maintain the particles in a suspended state. The coupling reaction was terminated by pelleting the particles with a magnet and removing the supernatant. The supernatant was saved for protein coupling efficiency analysis. The particles were then washed three times with wash diluent (0.05% Tween 20 in 10 mM PBS, pH7.4) and resuspended with 1 ml of storage diluent (0.1% BSA, 0.05% Tween 20 in 10 mM PBS, pH 7.4) to give a solids concentration of 1% by weight.

Measurement of the optical density at 280 nm of the first supernatant indicated that 740 μg of the antibody was bound to the particles.

Murine monoclonal anti-CKMB antibody (BiosPacific, #2717) was bound to the anti-murine IgG-coated magnetizable particles by mixing 250 μL of anti-CKMB at 0.74 mg/mL in PBS, pH 7.4 with 100 μL of 1% wt./vol. suspension of anti-murine IgG coated magnetizable particles. The mixture was incubated for 2 hours at room temperature (RT). The particles were then washed three times with PBS, 0.1% BSA, 0.05% Tween 20, pH 7.4. After the third wash, the particles were stored in 2 mL of wash buffer to give a solids concentration of 0.1% by weight.

3. PREPARATION OF ANTI CKMM-DEXTRAN-STREPTAVIDIN

1.) SMCC/AMINODEXTRAN: 1 mg in 1 mL PBS pH 7.4 of aminodextran (Molecular Probes, #D-7145) was mixed with 16.7 μL of SMCC (Pierce, succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, #223221) at 2 mg/mL in dimethylforamide. The mixture was allowed to react for 2 hours at RT. SMCC-labeled aminodextran was purified on a Sephadex® G-25 column previously equilibrated in PBS, pH 7.4. The column was run using the same buffer.

2.) SATA/ANTI-CKMM: 1 ml of anti-CKMM (BiosPacific, #G31520015) at 2mg/ml in PBS, pH 7.4 was mixed with 28.9 μL of

SATA (Pierce, N-succinimidyl-S-acetylthioacetate, #26102) at 2 mg/mL in dimethylforamide. The mixture was allowed to react for 2 hours at RT. The SATA-labeled antibody was purified on a Sephadex® G-25 previously column equilibrated in PBS, pH 7.4. The column was run using the same buffer.

3.) SATA/STREPTAVIDIN: 1 mL of streptavidin (Scripps Labs, #S1214) at 0.15 mg/mL in PBS, pH 7.4 was mixed with 5.8 µL of SATA at 2 mg/mL in dimethylforamide. The mixture was allowed to react for two hours at room temperature. The SATA-labeled streptavidin was purified on a Sephadex® G-25 column in PBS, pH 7.4.

4.) PROTEIN CONJUGATION: 1.0 M Hydroxylamine, pH 7, was added to a mixture containing SMCC-aminodextran (1 mg), SATA-anti CKMM (2 mg), and SATA-streptavidin (0.15 mG) in 7.5 mL of PBS, pH 7.4, to a final hydroxylamine concentration of 100 mM. The mixture was reacted overnight at 4°C. The reaction was stopped by addition of n-ethylmaleimide at a final concentration of 1 mM and incubated for 15 minutes at RT. The conjugate was then purified with a Bio Gel® A5m column. PBS, pH 7.4, was the eluting buffer.

4. PREPARATION OF STREPTAVIDIN / ANTI MURINE IgG COATED MAGNETIZABLE PARTICLES

50 µL of a 10% wt./vol suspension of magnetizable particles (Bangs Labs, #M0007700CN, 0.77 µm diameter, latex encapsulated, 41% magnetite) were washed 3 times with PBS pH 5.5 buffer. The particles were then pelleted with a magnet. One ml containing 100 µg streptavidin (Scripps Labs, #S1214) and 400 µg of anti-murine IgG (Sigma, #M-3534) in PBS, pH 5.5 was mixed with the particles. Twenty mg of carbodiimide (EDC, Pierce, #22980) was added to the particle/protein mixture and reacted for 2 hours at RT with constant mixing and a 5 minute sonication every 30 minutes. The particles were then pelleted with a magnet and washed 3 times with 1.0 mL volumes of PBS, 0.1% BSA, 0.05% Tween 20, pH 7.4. After the third wash the particles were stored in 1 mL of wash diluent to give a solids concentration of 0.5% by weight.

Murine monoclonal anti-CKMB antibody (BiosPacific, #2717) was bound to the anti-murine IgG/streptavidin-coated

particles by mixing 1.3 mL of anti-CKMB at 0.74 mg/mL in PBS, pH 7.2 with 1 mL of 0.5% wt./vol. suspension of the coated particles. The mixture was incubated 2 hours at RT with constant mixing and a 5 minute sonifcation step every 30 minutes. The particles were then washed 3 times with PBS, 0.1% BSA, 0.05% Tween 20, pH 7.2. After the third wash, the particles were resuspended and stored in 1 mL of wash buffer to give a solids concentration of 0.5% by weight.

5. PREPARATION OF ANTI-CKMM-HRP

1.) SATA/ANTI-CKMM: 2.0 mg of anti-CKMM (BiosPacific, #G31520015) in 1.0 mL of PBS, pH 7.4 was mixed with 47 µL of a 2 mg/mL solution of SATA (Pierce, #26102) in dimethylformamide. The mixture was allowed to react for 3 hours at room temperature. The SATA-labeled antibody was purified on a Sephadex® G-25 column equilibrated in PBS, pH 7.4.

2.) SMCC/HRP: Horseradish peroxidase (HRP,2.5 mg Sigma, #P-8415) in 1.0 mL of PBS, pH 7.4 was mixed with 42 µL of a 10 mg/mL solution of SMCC (Pierce, #223221) in dimethylformamide. The mixture was reacted for 3 hours at room temperature. The SMCC-labeled HRP was purified on a Sephadex® G-25 column equilibrated in PBS, pH 7.4.

3.) CONJUGATION OF ANTI-CKMM TO HRP: Hydroxylamine was added at a final concentration of 0.1 M to a 5 mL solution containing 2 mg of SATA-anti-CKMM and 2.5 mg of SMCC-HRP in PBS, pH 7.4. The mixture was allowed to react overnight at 4°C. The conjugation reaction was stopped by adding n-ethylmaleimide to a final concentration of 1 mM and incubating for 15 minutes at room temperature. The anti-CKMM-HRP conjugate was purified on a BioGel® A5m column equilibrated in PBS, pH 7.4.

6. MAGNETIZABLE PARTICLE MEDIATED IMMUNOASSAY

A. First Protocol

a.) Disks were pre-coated with anti-CKMM as follows: 400 µL of the (anti-CKMM/streptavidin)-dextran conjugate in assay buffer (1 mg/mL BSA, 0.05% Tween 20, PBS, pH 7.2) was

placed on the surface of the acrylic disk having 32 (1 mm round) spots of adsorbed biotinylated BSA. The disks were incubated overnight at RT and then washed 4 times with wash buffer.

b.) Magnetizable particle binding step: 400 μL of a 0.05% suspension of magnetizable particles coated with anti-CKMB in assay buffer were mixed with 100 μL of CKMB-containing samples in PBS, pH 7.4. The mixture was incubated for 10 minutes at room temperature with constant mixing to maintain the particles in suspension.

c.) Disk binding step: The particle/CKMB suspension was transferred to disks 400 pre-bound with anti-CKMM. The disks 400 (Fig. 7), were then mounted on a spindle 402 over a 0.91 inch ring magnet 404 (Dexter, Alnico 8, #8F508) poled parallel to the thickness of the magnet. A motordrive 406 attached to the spindle 402 rotated the disk at a velocity of about 1 revolution per second for 1 second in each direction. The ring magnet was on a moveable platform 408 that positioned the magnet directly under the disk. When raised, the outer edge of the magnet 404 was positioned directly under the reagent spots 410 on the upper disk surface. As best seen in Fig. 8, the magnetic lines of flux (shown as lines 412) are concentrated at the edge of the magnetic and passes directly through the reagent spots 410. Operation of the system, including piston and cylinder 414 for raising the platform 408, was controlled by a personal computer and associated interface.

d.) Detection

i. Interferometric Thickness Measurement

After disc preparation was completed as described above, the disc was mounted in an interferometric measuring system as described in copending PCT application US94/07184. The system is shown schematically in Fig. 8A of the present application. The discs were rotated in the beams of the interferometer at a speed of 100 revolutions per second. The electronic measurement was taken from signal point A at th output of differential amplifier 160 in Fig. 8A. The analog signal at A was converted into a digital signal by signal

processor 167. The digital conversion hardware and processing software used were the AT-MIO-16X DAQ acquisition board and the LabVIEW data processing software, both from National Instruments Corporation (Austin, Texas).

The discs were prepared with 32 immuno-reactive spots. As a result, the information being measured was modulated to a frequency equal to 32 times the motor rotation speed (in this case 3.2 KHz). The output signal for the assay was read as the fourier transform component at 32 times the motor rotation frequency. The interferometer measured the phase difference between the two light beams in the device after they had traveled two different paths, one traversing immunoreacted spots and the other not traversing such a spot. The phase of the beam which passes through the immunoreacted spot is shifted due to the increase in the index of refraction caused by the presence of the magnetizable particles. The index of refraction of the material bound to the immunoreactive spots increases as more magnetic particle complexes are bound to the spot, thus serving as the source of signal modulation measured by the detection device.

ii. Light Absorbance (Transmission Loss) Measurement

In order to make a transmission loss measurement, the interferometer and electronics of Fig. 8A were modified as shown in Fig. 8B. The goal of the measurement was to quantify the loss in total light reaching the detectors. In the magnetizable particle assay, the particles present on the immunoreactive spots absorb and scatter the laser beam incident on the spot. This effect was measured as a reduction in the amount of light reaching the detectors 142 and 145 in Fig. 8B. The simplicity of the modification between the interferometric and transmission loss configurations allowed the measurement of both interference (i.e. phase shift) and transmission loss without removing the disc from the motor shaft betw en measurements.

As shown in Fig. 8B, in order to measure the power of the light reaching the detectors 142 and 145, summing amplifier 160b was added. This created a signal which is

proportional to the power reaching th detectors. When measuring transmission loss, signal C was connected to the signal processor. As with interferometry, measurement is made by using the fourier transform component at the signal frequency 32 times the motor rotation frequency.

In order to measure only the power of the beam reaching the detectors 142 and 145, and not be affected by interference effects taking place at beam splitter 175 (Fig. 8B), it was necessary to remove optical interference from the system by removing one of the beams. This was performed by inserting a beam block 162b into the path of the beam transmitted through beam splitter 162. Note that the beam does not travel beyond the beam block and thus only one beam travels through the spots on the disc.

The signals which result for transmission loss measurement are shown in Figs. 8C and 8D. When there is no signal lost in traversing the disc (corresponding to no magnetizable particles bound to the spots), the signal output from amplifier 160b is a pure DC signal proportional to the laser power (Fig. 8C). When magnetizable particles are bound to the spots, the laser beam is absorbed and scattered by the particles resulting in less light reaching the detectors. The signal resulting is shown in Fig. 8D. The AC portion of this signal represents the transmission loss signal and has a fundamental frequency at 32 times the motor rotation frequency.

B. Second Protocol

a.) CKMB Standard (50 µl) was mixed with 50 µl of anti-CKMM-HRP conjugate. 200 µl of the magnetizable particle conjugate was then added. The mixture was then incubated for 10 minutes at RT with constant mixing.

b.) The disks were placed on, and the magnetic mixer, and the CKMB/magnetizable particle mixture was placed on the disk and pelleted by the magnet for a total of 4 minutes.

c.) Disks were gently rinsed/swirled in wash diluent for total of two washes.

d.) One tablet of OPD (o-phenylenediamine dihydrochloride, Sigma #P4664) was dissolved in 37 ml of 0.1 M citrate phosphate buffer, pH 5.0. Just prior to application, 37 µl of 30% hydrogen peroxide was added to OPD solution.

e.) The mixture containing OPD and hydrogen peroxide (300 µl) was added onto each disk. The disks were incubated for 10 minutes at RT.

f.) At the end of the 10 min. incubation, 300µl of 2N sulfuric acid was added onto each of the disks. The colored supernatant was then aliquoted into a microplate. A microplate reader (Emax, Molecular Devices) was used to read absorbance at 490 nm.

5. RESULTS

Test results from the first protocol are shown in Fig. 9 and (thickness measurement) in Fig. 10 (transmission loss). In both cases, the ability to quantitate various levels of CKMB concentrations is demonstrated. An example of the second protocol where a specific binding pair captures a constant amount of magnetizable particles is shown in Fig. 11. Using an anti-CKMM-HRP conjugate as label, the second protocol also quantitated CKMB levels ranging from zero to 600 ng/ml.

Although the foregoing invention has been described in some detail by way of illustration and example, for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An assay for determining the presence of an analyte which is a member of a specific binding pair (SBP) in a sample, said assay comprising:

exposing the sample to a solid phase surface having a reaction zone comprising one member of a SBP in the presence of magnetizable particles having one member of a SBP bound thereto;

applying a magnetic field aligned with the reaction zone to attract the magnetizable particles thereto, wherein magnetizable particles are bound across the reaction zone; and

determining the amount of analyte in the sample based on the amount of the magnetizable particles bound in the reaction zone.

2. An assay as in claim 1, wherein the analyte is ligand, the reaction zone comprises anti-ligand, and the magnetizable particles have anti-ligand bound thereto, whereby the magnetizable particles are non-competitively bound to the reaction zone.

3. An assay as in claim 2, wherein anti-ligand bound to the magnetizable particles binds a different epitope on the ligand than does the anti-ligand in the reaction zone.

4. An assay as in claim 1, wherein the analyte is ligand, the reaction zone comprises anti-ligand, and the magnetizable particles have ligand bound thereto, whereby the magnetizable particles are competitively bound to the reaction zone.

5. An assay as in claim 1, wherein the analyte is ligand, the reaction zone comprises ligand, and the magnetizable particles have anti-ligand bound thereto, whereby the magnetizable particles are competitively bound to the reaction zone.

6. An assay as in claim 1, further comprising interrupting the aligned magnetic field and removing non-bound magnetizable particles prior to determining the amount of analyte in the sample.

7. An assay as in claim 6, wherein the step of removing non-bound particles includes applying a second magnetic field which draws non-bound particles away from the reaction zone.

8. An assay as in claim 1, wherein the amount of particles is measured interferometrically or spectrophotometrically.

9. An assay for determining the presence of an analyte which is a member of a specific binding pair (SBP) in a sample, said assay comprising:

exposing the sample to a solid phase surface having a reaction zone comprising one member of a SBP in the presence of magnetizable particles having one member of at least two different SBP's bound thereto, wherein a first particle-bound SBP member binds directly or indirectly to the solid phase SBP member and a second particle-bound SBP member binds directly or indirectly to the analyte;

incubating the magnetizable particles with the sample for a time sufficient to permit binding between the second SBP member and the analyte to occur;

applying a magnetic field across the reaction zone to enhance the rate of migration of the magnetizable particles, wherein articles will be bound within the reaction zone to immobilize analyte bound to the second SBP member; and

detecting the presence of analyte bound to the reaction zone.

10. An assay as in claim 9, further comprising exposing the sample to a labeled SBP member which binds to the analyte prior to applying the magnetic field, wherein detecting the analyte compris s detecting the label.

11. An assay as in claim 9, wherein the solid phase is a disk having a plurality of annularly spaced-apart reaction zones formed over one flat face.

12. An assay as in claim 11, wherein the magnetic field has an annular geometry corresponding to the layout of reaction zones on the disk.

13. A method as in claim 11, wherein the reaction zone has an area of at least 0.1 mm^2.

14. An assay as in claim 9, wherein the reaction zone SBP member is avidin and the first particle-bound SBP member is biotin.

15. An assay as in claim 9, wherein the second particle-bound SBP member is anti-analyte.

16. An improved non-competitive binding assay of the type wherein analyte is bound to a solid phase surface by a first antibody and detected by a labeled second antibody specific for a monomeric epitope on the analyte, wherein the improvement comprises employing a magnetizable particle as the label and applying a magnetic field aligned with a reaction zone on the solid phase surface to enhance the binding rate of analyte to the first antibody, whereby magnetizable particles are bound to the solid phase based on binding of analyte to the particle.

17. An improved assay as in claim 16, further comprising determining the amount of analyte in a sample based on the amount of magnetizable particles bound to the solid phase.

18. An improved assay as in claim 17, further comprising interrupting th aligned magnetic field and removing non-bound magnetizable particles prior to determining the amount of analyte in the sample.

19. An improved assay as in claim 18, wherein the step of removing non-bound magnetizable particles includes applying a second magnetic field which draws non-bound particles away from the reaction zone.

20. An improved assay as in claim 16, wherein the first antibody is bound within a substantially flat reaction zone on the solid phase surface.

21. An assay for determining the presence of an analyte which is a member of a specific binding pair (SBP) in a sample, said assay comprising:

exposing the sample to a solid phase surface having a reaction zone comprising one member of a SBP in the presence of magnetizable particles having a SBP member bound thereto, wherein the magnetizable particles bind to the analyte in a liquid phase;

applying a magnetic field which is not aligned with the reaction zone to separate the magnetizable particles from unbound analyte;

resuspending the magnetizable particles in a reaction medium,

exposing the reaction medium to the solid phase having a reaction zone comprising an SBP member which binds directly or indirectly to the analyte bound to the magnetizable particles;

applying a magnetic field aligned with the reaction zone to attract the magnetizable particles thereto;

determining the presence of analyte based on the binding of magnetizable particles to the reaction zone.

22. An assay for determining the presence of at least two different analytes in a sample wherein each is a member of a specific binding pair (SBP), said method comprising:

reacting the sample with first magnetizabl particles bound to (1) a first SBP member which binds to a first analyte and (2) a first capture receptor;

reacting the sample with second magnetizable particles bound to (1) a second SBP member which binds to a second analyte and (2) a second capture receptor;

exposing the sample to a solid phase having a first reaction zone comprising first capture ligand which binds to the first capture receptor and a second reaction zone comprising second capture ligand which binds to the second capture receptor;

applying a magnetic field aligned with the reaction zones to attract the magnetizable particles thereto; and

determining the presence of the first and second analytes based on binding to said analytes within their respective reaction zones.

23. An assay as in claim 22, wherein the magnetic field is periodically disrupted to permit non-specifically bound particles to migrate in a direction not aligned with the magnetic field.

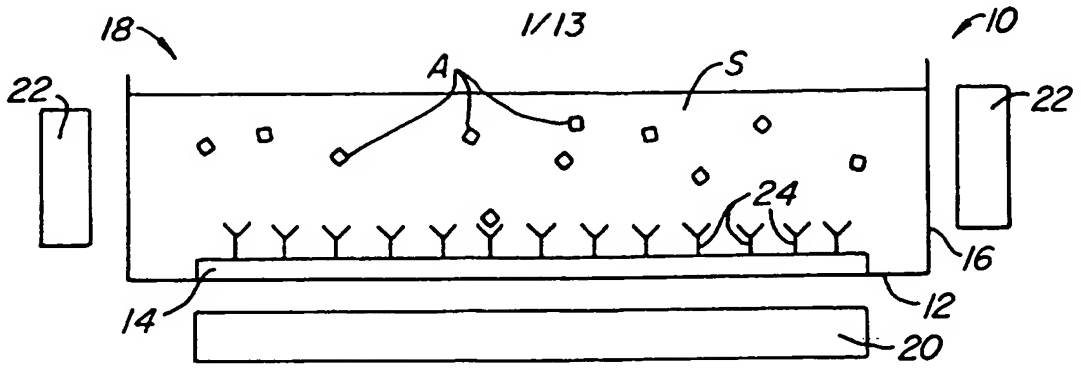


FIG. 1A.

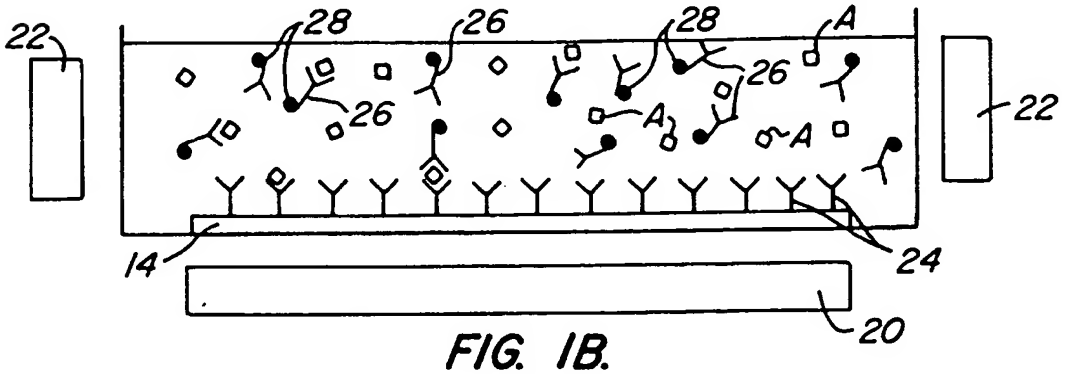


FIG. 1B.

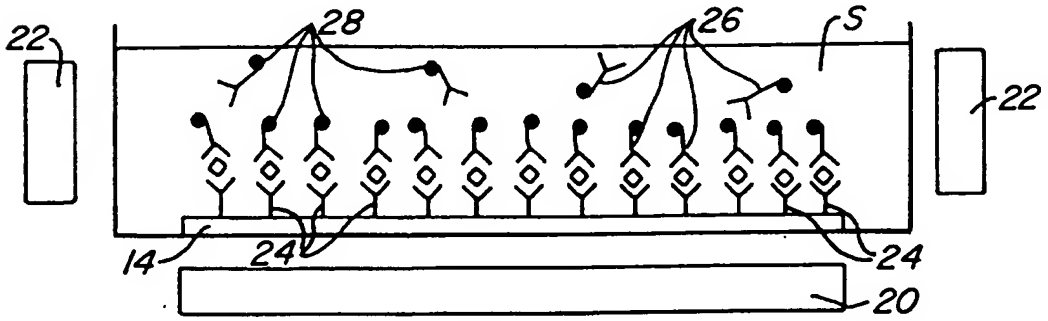


FIG. 1C.

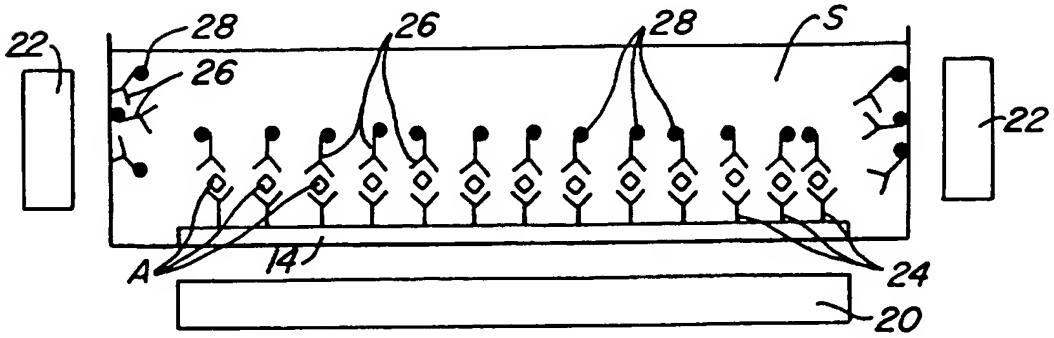


FIG. 1D.

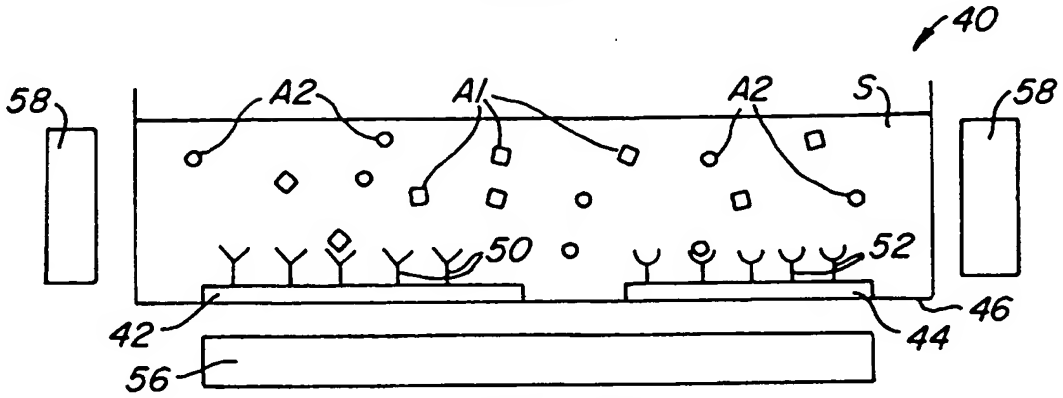


FIG. 2A.

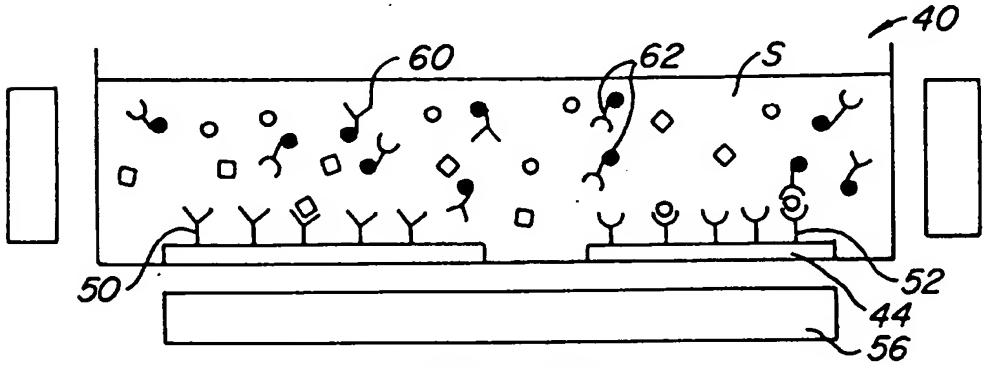


FIG. 2B.

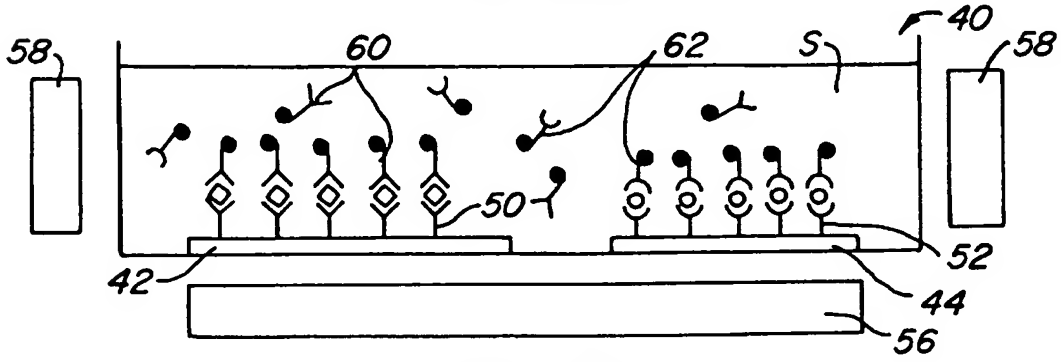


FIG. 2C.

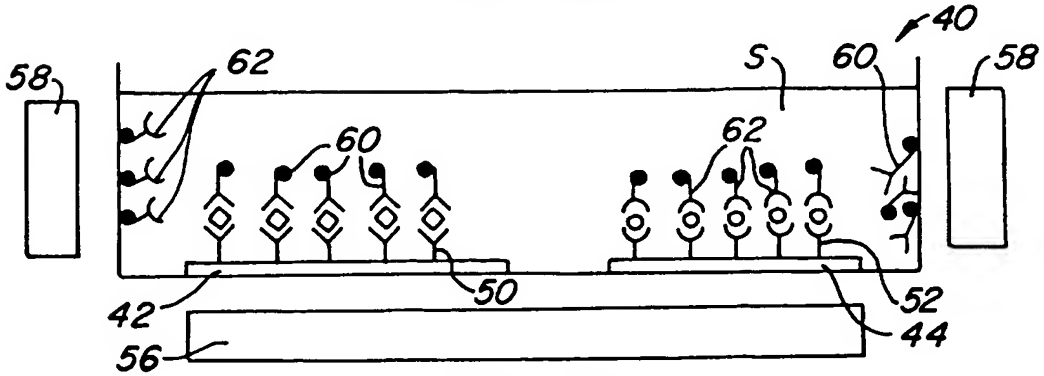


FIG. 2D.

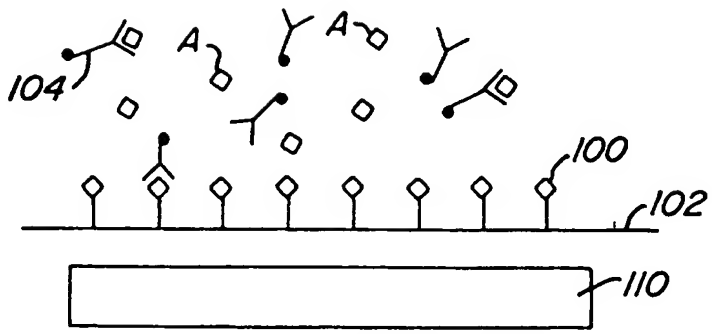


FIG. 3A.

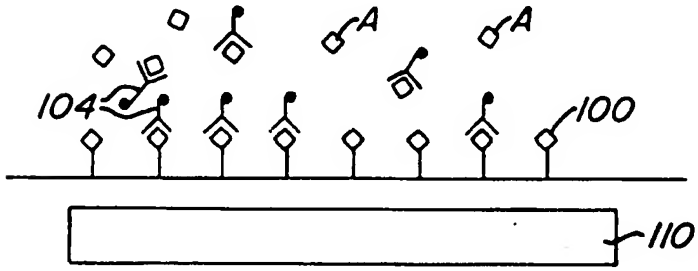


FIG. 3B.

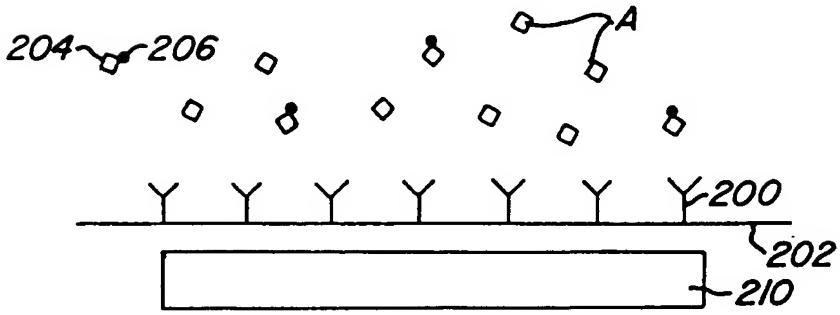


FIG. 4A.

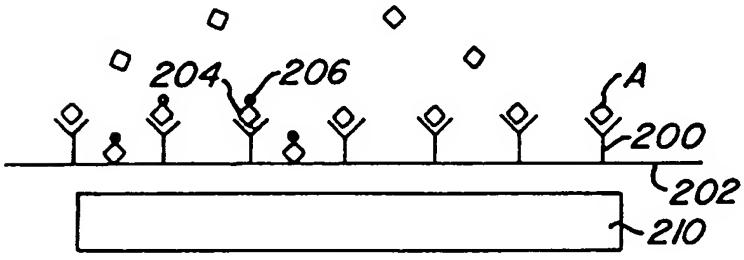


FIG. 4B.

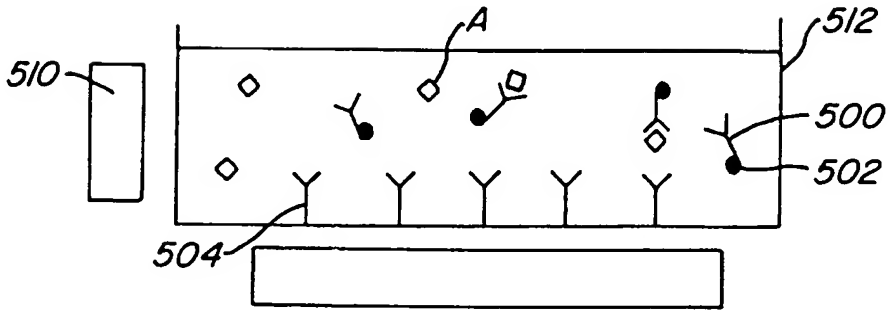


FIG. 5A.

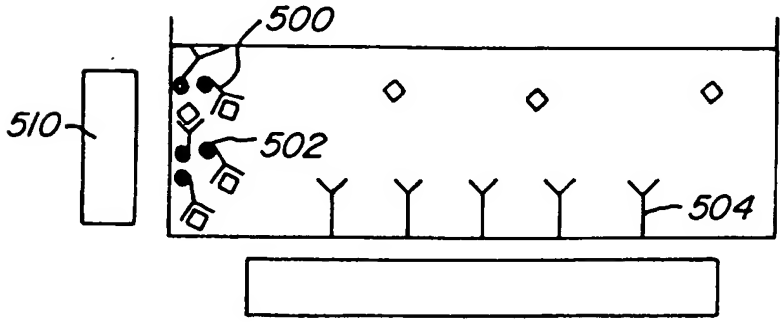


FIG. 5B.

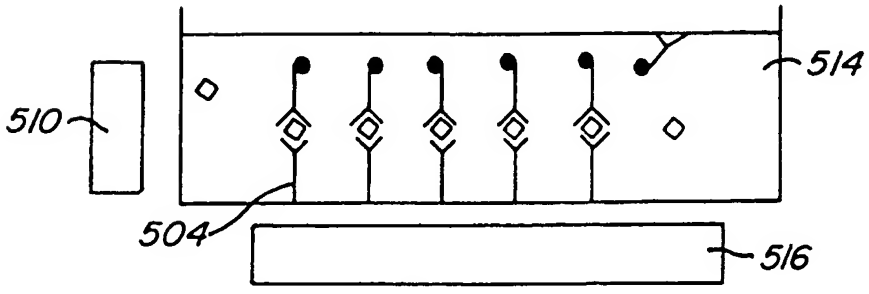


FIG. 5C.

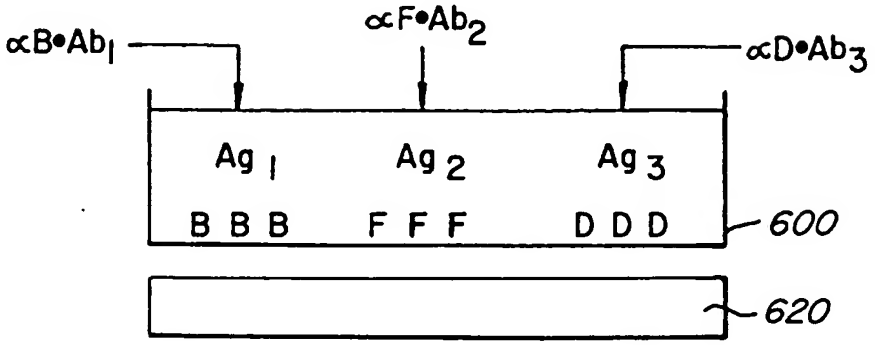


FIG. 5D.

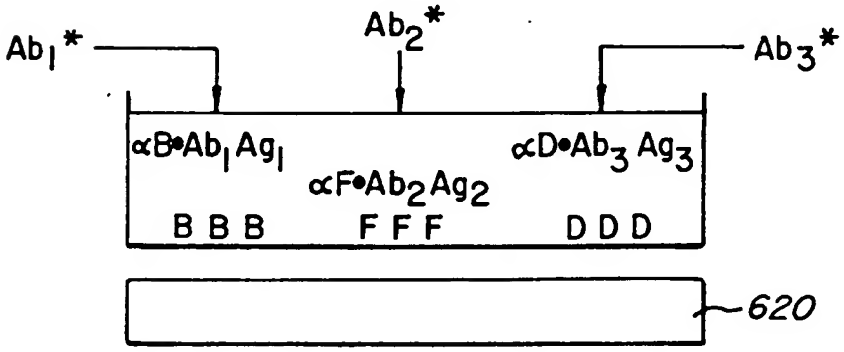


FIG. 5E.

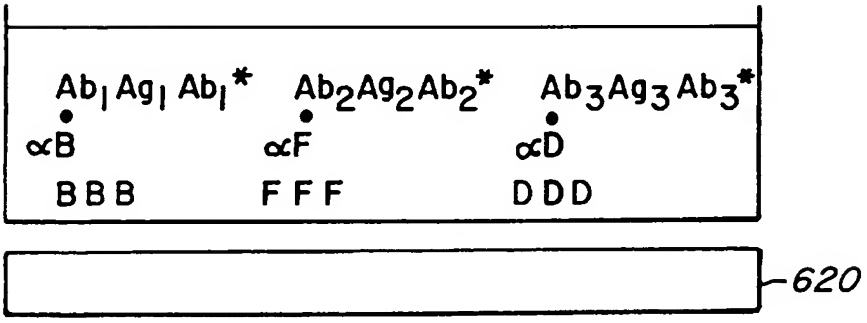


FIG. 5F.

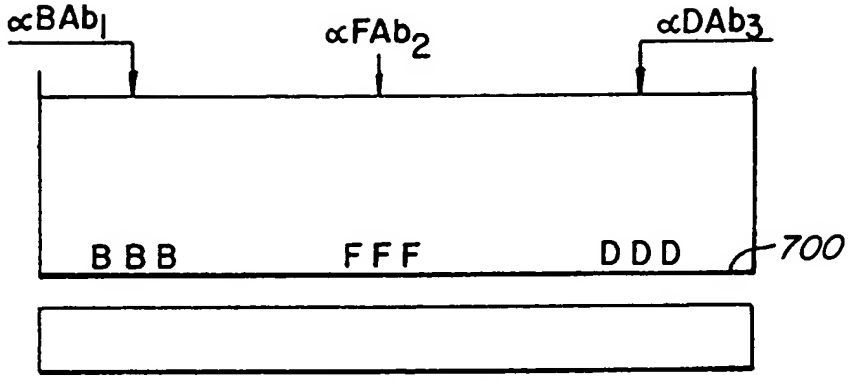


FIG. 5G.

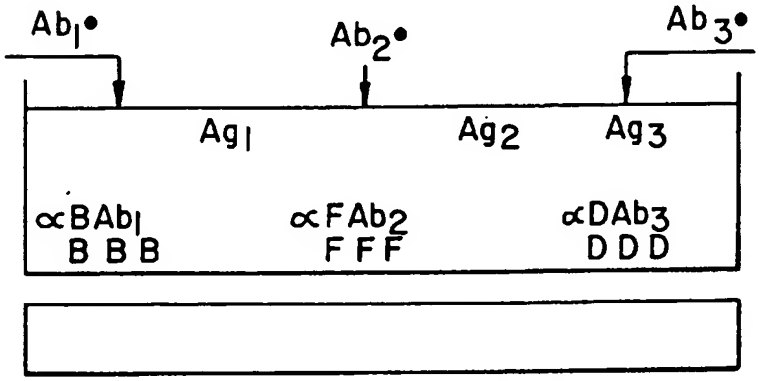


FIG. 5H.

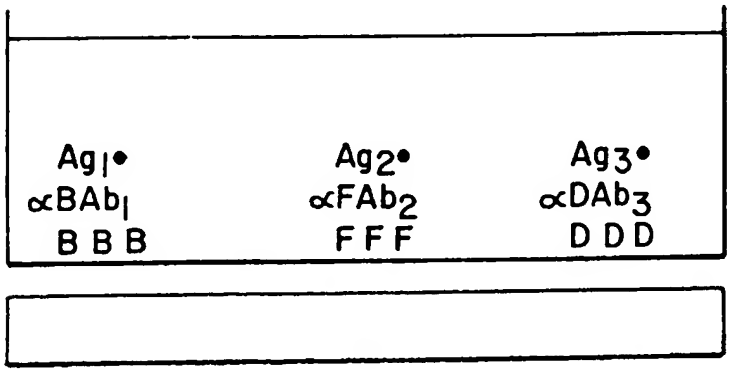


FIG. 5I.

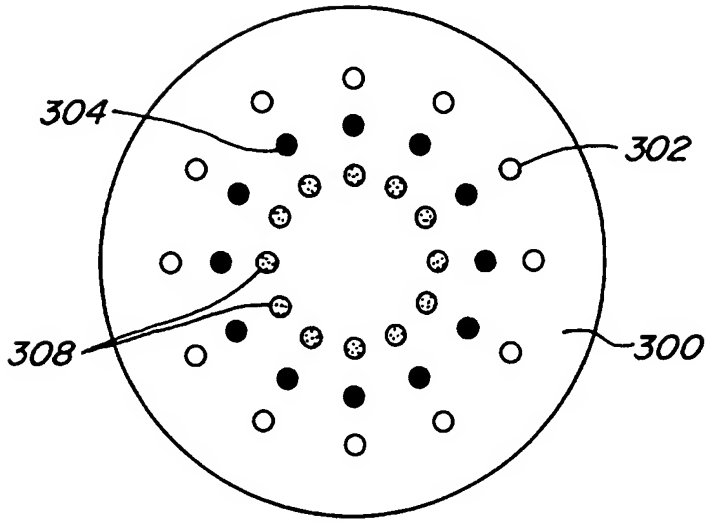


FIG. 6A.

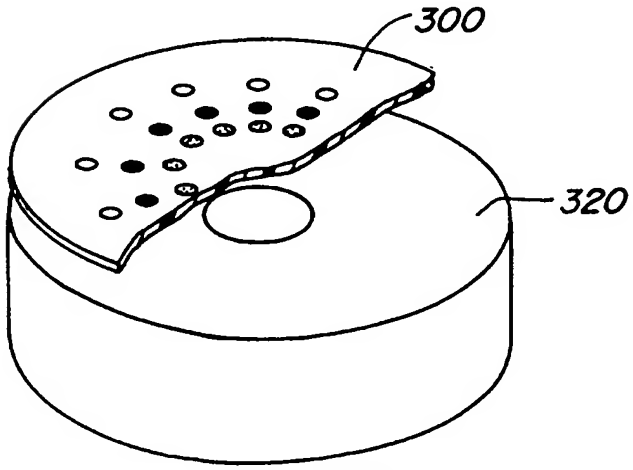


FIG. 6B.

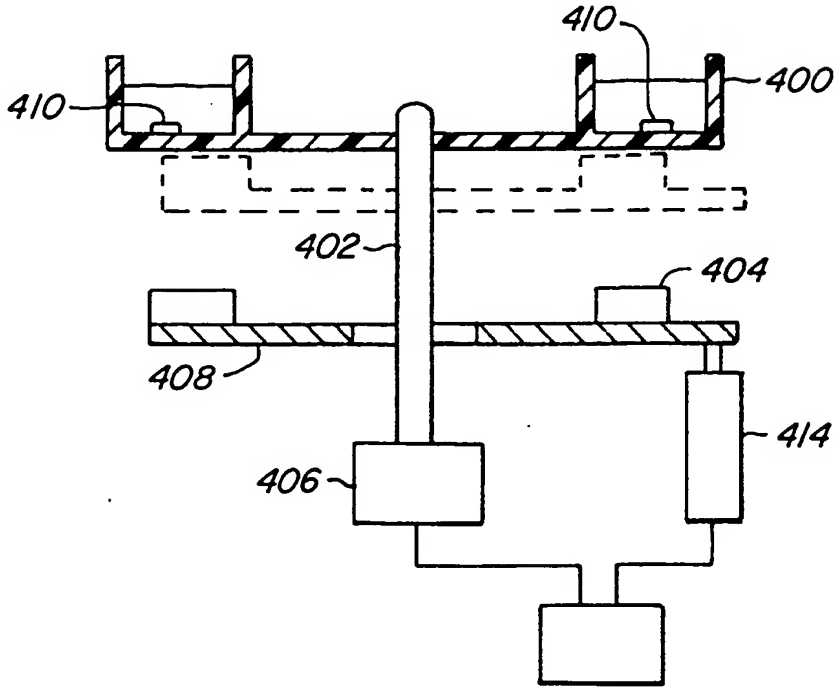


FIG. 7.

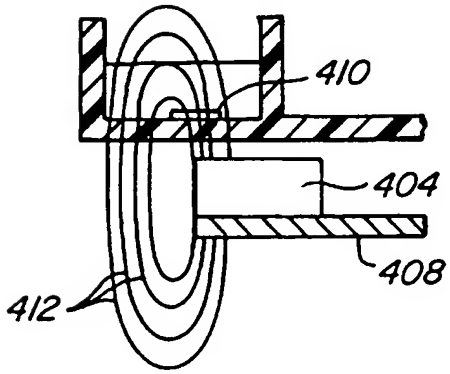
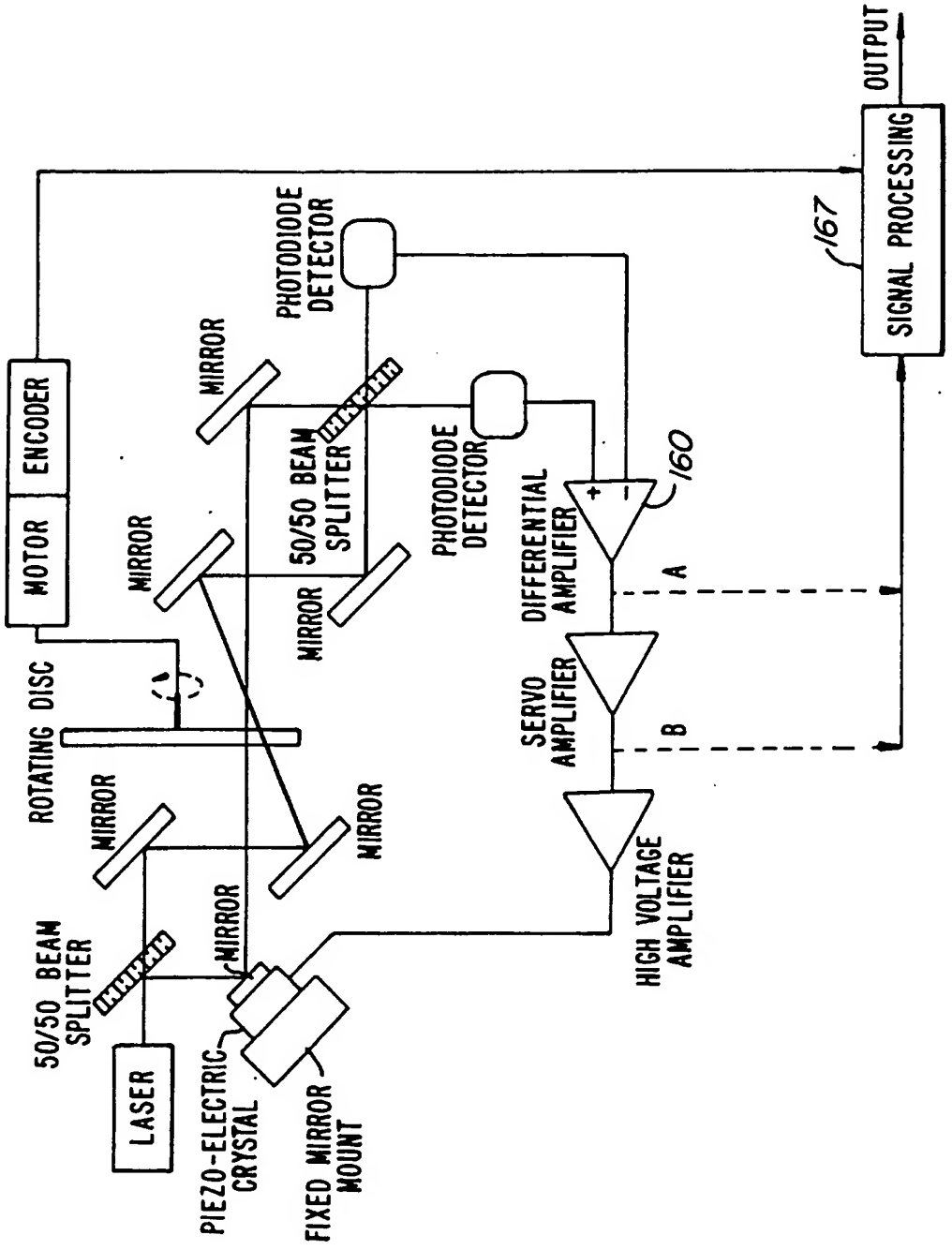


FIG. 8.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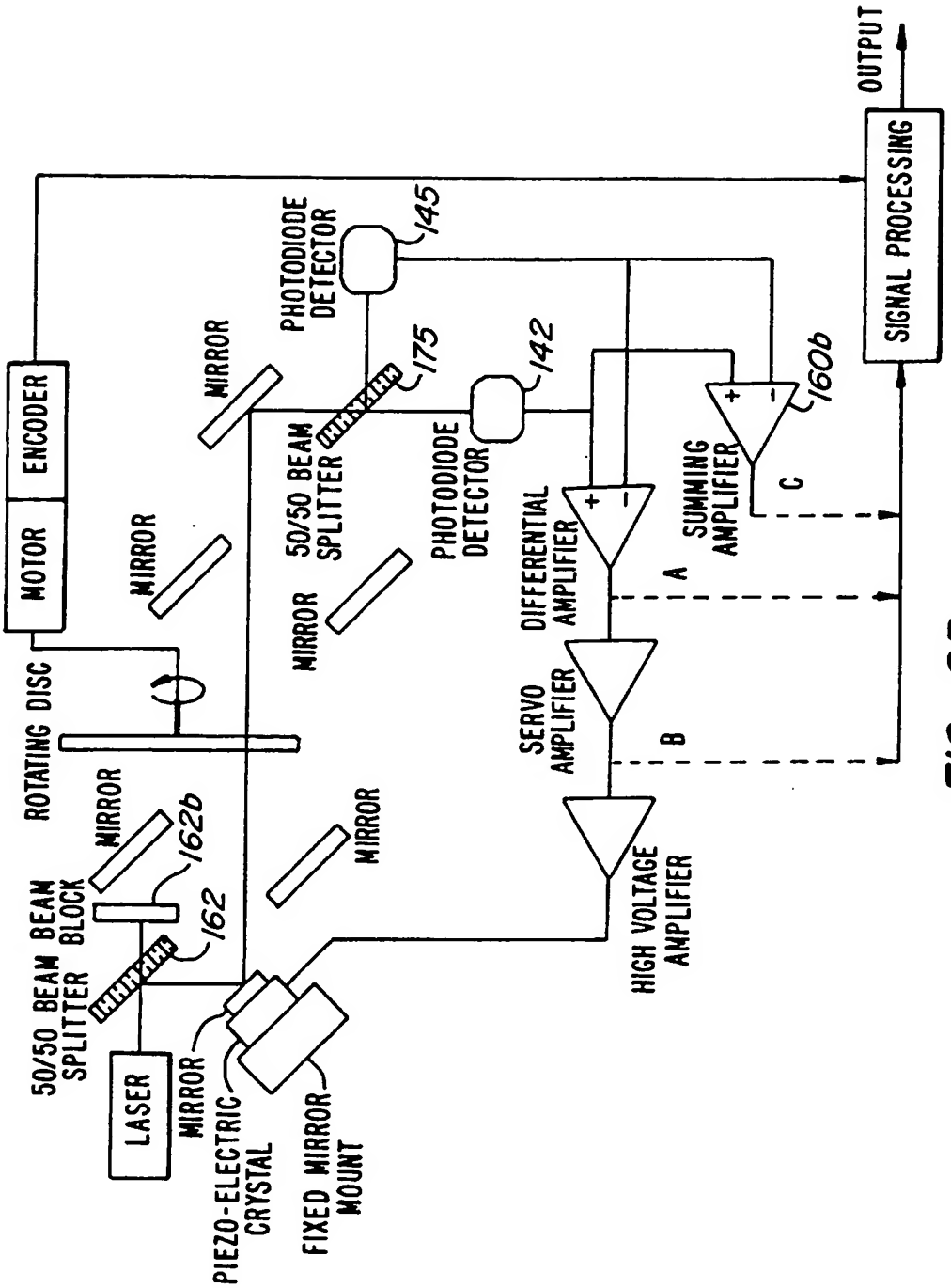
167
PHOTODIODE DETECTOR
MIRROR
50/50 BEAM SPLITTER
PHOTODIODE DETECTOR
DIFFERENTIAL AMPLIFIER
160
ENCODER
MOTOR
MIRROR
MIRROR
A
ROTATING DISC
SERVO AMPLIFIER
B
MIRROR
MIRROR
HIGH VOLTAGE AMPLIFIER
50/50 BEAM SPLITTER
MIRROR
LASER
PIEZO-ELECTRIC CRYSTAL
FIXED MIRROR MOUNT

FIG. 8A.


OUTPUT
SIGNAL PROCESSING
PHOTODIODE DETECTOR
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MIRROR
175
142
160b
ENCODER
MOTOR
50/50 BEAM SPLITTER
PHOTODIODE DETECTOR
SUMMING AMPLIFIER
C
MIRROR
MIRROR
DIFFERENTIAL AMPLIFIER
A
ROTATING DISC
SERVO AMPLIFIER
B
MIRROR
162b
MIRROR
BEAM BLOCK
162
HIGH VOLTAGE AMPLIFIER
50/50 BEAM SPLITTER
LASER
MIRROR
PIEZO-ELECTRIC CRYSTAL
FIXED MIRROR MOUNT

FIG. 8B.

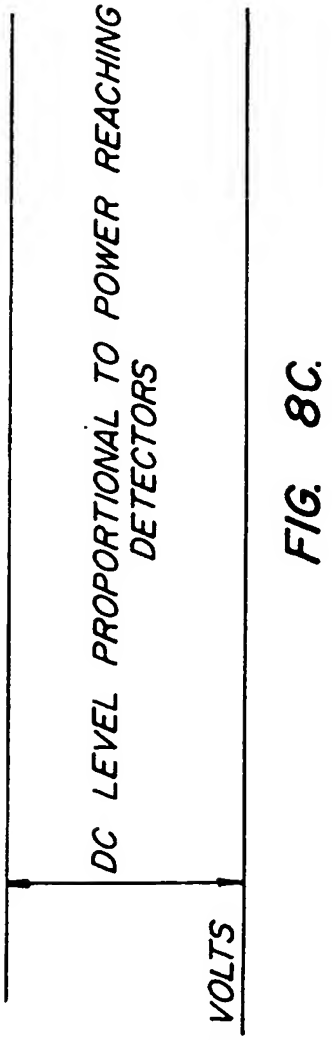
DC LEVEL PROPORTIONAL TO POWER REACHING DETECTORS
0 VOLTS

FIG. 8C.

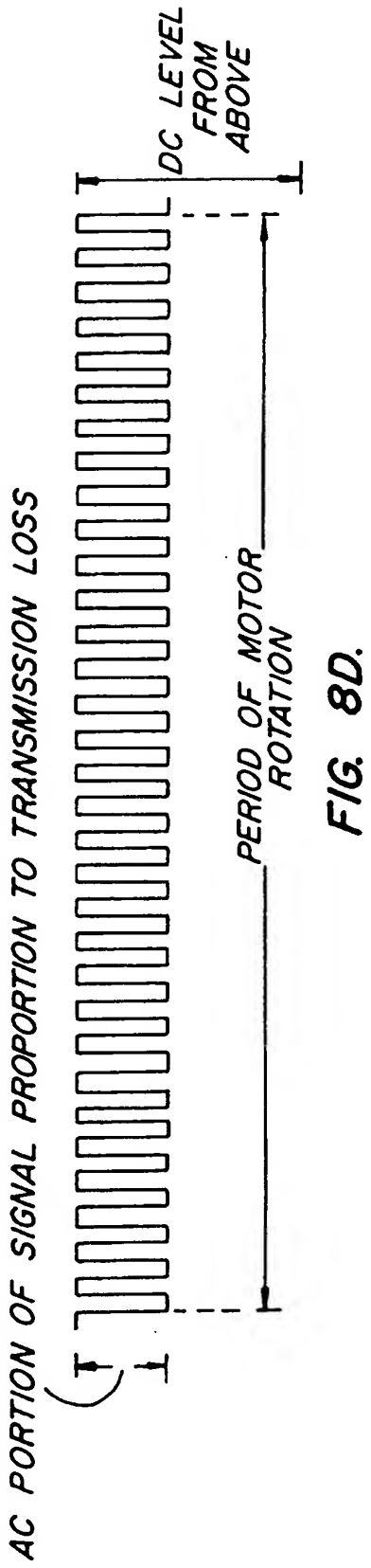
DC LEVEL FROM ABOVE
PERIOD OF MOTOR ROTATION
AC PORTION OF SIGNAL PROPORTION TO TRANSMISSION LOSS

FIG. 8D.

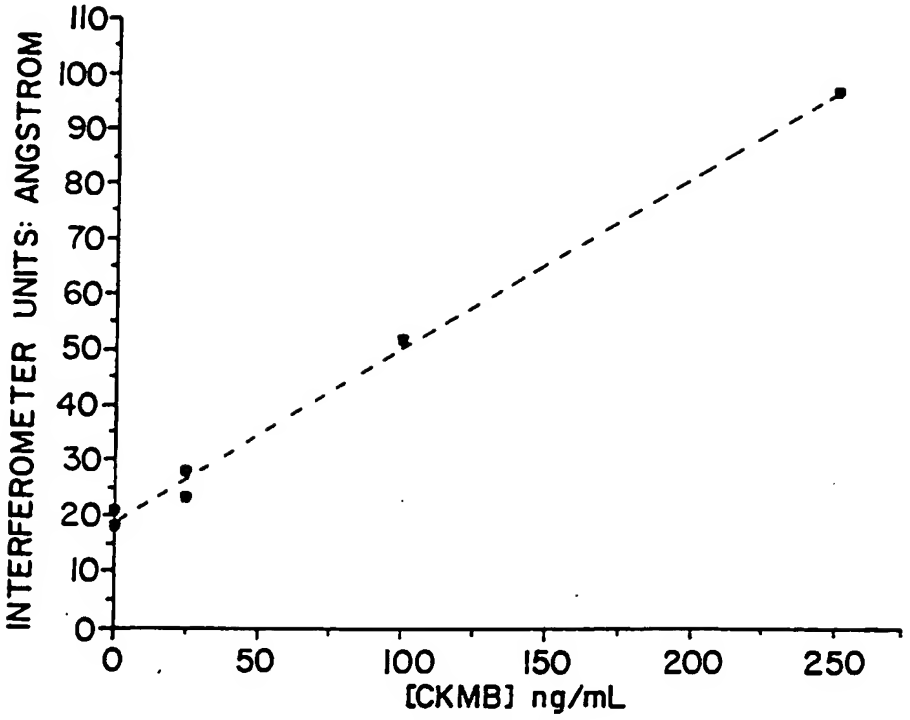
INTERFEROMETER UNITS: ANGSTROM
110
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
[CKMB] ng/mL

FIG. 9.

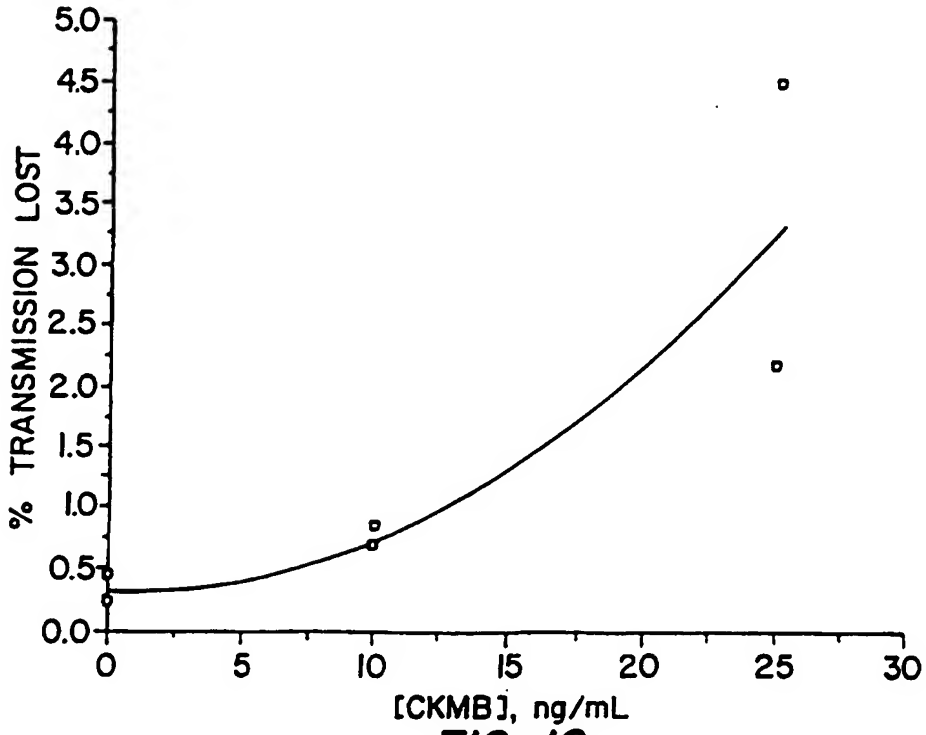
% TRANSMISSION LOST
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
[CKMB], ng/mL

FIG. 10.

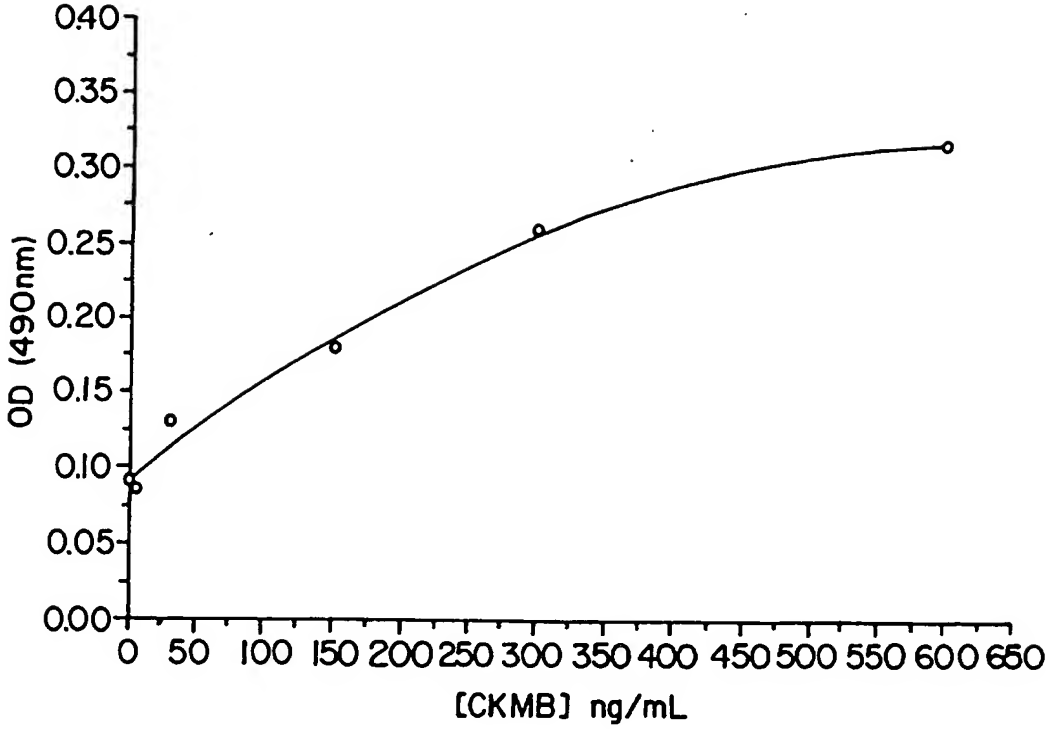
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD (490nm)
0 50 100 150 200 250 300 350 400 450 500 550 600 650
[CKMB] ng/mL

FIG. 11.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/10680

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/553
US CL :436/526

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/518, 524, 526, 528, 531, 536, 538, 540, 805, 806

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

NONE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	JP, A, 63-108264 (NIPPON TELEG & TELEPH CORP) 13 May 1988, see abstract and figures.	1-3, 6, 7 ---------- 4, 5
X --- Y	EP, A, 0 351 857 (OLYMPUS OPTICAL CO., LTD.) 24 January 1990, see entire document, especially abstract, page 3, lines 26-28, and page 4, lines 18-20, 27-28, and 50-52.	1, 2, 4, 5 ---------- 3, 6-8, 21
Y	US, A, 5,252,493 (FUJIWARA ET AL.) 12 October 1993, see especially Figure 2(e) and column 21, lines 30-36.	6-8, 18-19, 21, 23
X ---- Y	JP, A, 3-189560 (OLYMPUS OPTICAL CO., LTD.) 19 August 1991, see abstract.	9, 15 --------- 10-14, 22, 23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 29 NOVEMBER 1995	Date of mailing of the international search report 04 JAN 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer SUSAN C. WOLSKI Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/10680

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, A, 63-302367 (NIPPON TELEG & TELEPH) 09 December 1988, see abstract, page 7, second full paragraph, page 8, first full paragraph, and figures.	11-13, 22, 23
Y	EP, A, 0 522 322 (OLYMPUS OPTICAL CO., LTD.) 13 January 1993, see entire document, especially page 2, lines 10-11 and 35-37, page 3, lines 10-19 and 38-40, and page 5, lines 4-7.	16-20